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Award Number: W81XWH-12-2-0134

TITLE: A Military-Relevant Model of Closed Concussive Head Injury:
Longitudinal Studies Characterizing and Validating Single and Repetitive mTBI

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE October 2015		2. REPORT TYPE Annual		3. DATES COVERED 30 Sep 2014 - 29 Sep 2015	
4. TITLE AND SUBTITLE A Military-Relevant Model of Closed Concussive Head Injury: Longitudinal Studies Characterizing and Validating Single and Repetitive mTBI				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-12-2-0134	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Deborah A Shear (PI), Dr. Frank C Tortella (Co-PI), Dr. Lai Yee Leung, Dr. Ying-Deng Bryant, CPT Andrea Mountney, Dr. Casandra Cartagena, Dr. Angela Boutte, Dr. Xi-Chun May Lu, CPT Wayne Johnson, Dr. Sindhu Madathil, Dr. Jenny Browning E-Mail: deborah.a.shear.civ@mail.mil				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Geneva Foundation 917 Pacific Ave, Suite 600 Tacoma, WA 98402				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Because of sports injuries, automobile accidents, falls, etc., and with the escalation of the use of improvised explosion devices (IEDs) by our enemies as witnessed in the most recent military conflicts in Iraq and Afghanistan, there has been an increased awareness of closed head concussions, also commonly referred to as the mild TBI (mTBI) injury. The prevalence of this type of closed-head brain injury, estimated as afflicting over 300,000 deployed soldiers or approximately 30% of all deployed troops, has distinguished it as the "signature injury" of these military conflicts. Despite the enormity of this medical problem, and recognition of the importance for the need to quickly and accurately diagnose the event in the face of a limited clinical presentation (i.e. no obvious wounds to the head), objective diagnostic tools and knowledge about what occurs in the brain following this type of injury are limited. Of equal concern is our lack of understanding the impact of multiple concussions on the brain and its consequences on the long term health of individuals. In order to address this problem, the WRAIR projectile concussive impact (PCI) model was developed under directive of the Combat Casualty Care Research Program (CCCRP). Provided in this Year 2 Annual Report are the results of our Phase I studies focused on characterizing the neuropathologic, molecular and neurobehavioral changes following a single concussive impact (PCI) injury. Additionally, this Report also includes data comparing the effects of a single concussive impact to repeated concussive impacts using the PCI model. Phase I studies have been completed and these results set the foundation for Phase II studies designed to evaluate the effects of repeated concussions that occur prior to and after the resolution of the healing profile for a single concussion.					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU		19b. TELEPHONE NUMBER (include area code)

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

Under the directive of the Combat Casualty Care Research Program (CCCRP) to establish a military-relevant model of concussive head injury, the proof-of-concept development of the WRAIR Projectile Concussive Impact (PCI) model of closed-head mTBI has been successfully completed. In addition, in collaboration with the Composites and Hybrid Materials Branch, Army Research Laboratory (Aberdeen) we have recently completed the development and implementation of custom-designed helmets combined with pressure sensor film analysis to detect the impact pressure distribution pattern both on the outer and inner helmet surface. The overall goal of the current proposal is to conduct longitudinal studies on the WRAIR PCI model following “SINGLE” or “REPEATED” PCI injuries in order to develop a more thorough understanding of the changes taking place at a cellular level following a single or multiple concussive events and to establish how those changes relate to clinically relevant mTBI behavioral and electrophysiological outcome metrics. Concussive head injury will be studied in the WRAIR PCI model using longitudinal and multi-modal designs to fully characterize the neuromotor, cognitive, emotional, and neuropathological evidence of brain injury. ***Phase I (SOW 1) studies will fully characterize the neuropathological, molecular and neurobehavioral changes following a “SINGLE” PCI injury. Phase II (SOW 2) studies will evaluate the cumulative effects of “REPEATED” PCI injuries based on outcome metrics defined in SOW 1.***

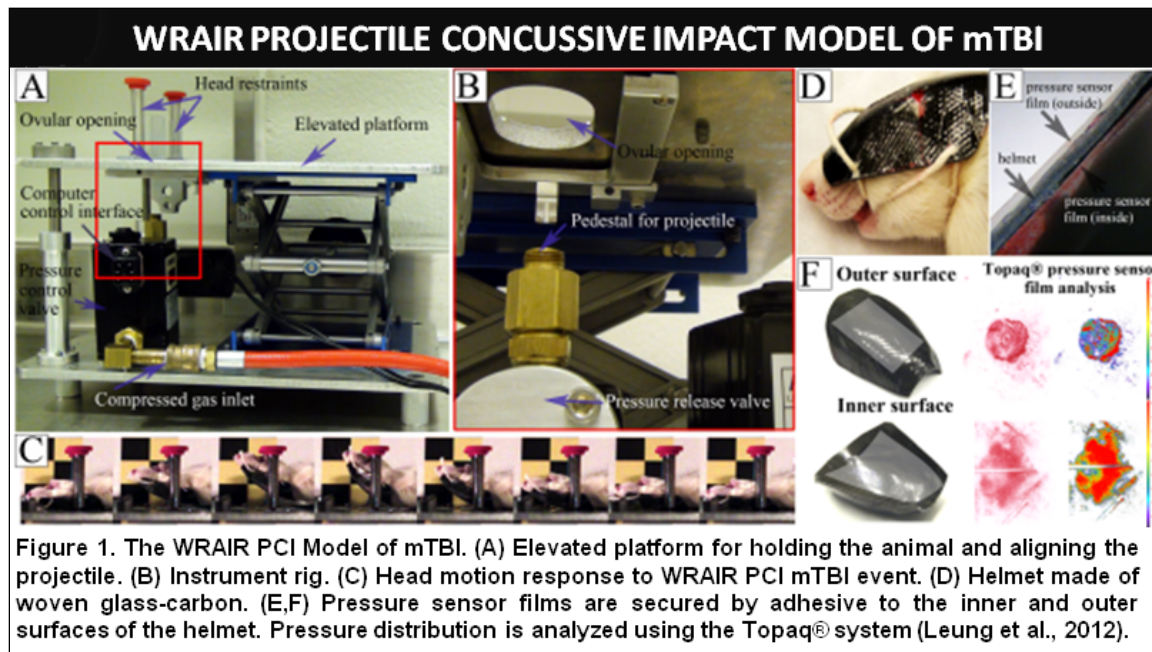
PT110545 TIMELINE FOR STATEMENT OF WORK	FY 2013	FY 2014	FY 2015	FY 2016
SOW 1: SINGLE PCI (Months 1-24)				
Task 1.0 Completion of all Regulatory Processes				
Task 1.1 Single PCI: Histopathology Profile				
Experiment 1.1.1. Diffuse Axonal Injury				
Experiment 1.1.2. Glial Response				
Experiment 1.1.3. BBB Permeability				
Task 1.2 (Months 6-18) Single PCI: Molecular Profile				
Experiment 1.2.1. Molecular Changes				
Experiment 1.2.2. Protein Biomarkers				
Experiment 1.2.3. Bioenergetic Profile				
Task 1.3 Single PCI: Neurobehavioral Profile				
Task 1.4 Single PCI: qEEG Profile				
SOW 2: REPEATED PCI (Months 24-48)				
Task 2.1. Compare Effects Repeated vs. Single PCI				
Experiment 2.1.1. Effects of 2nd PCI prior to resolution of healing profile (HP)				
Experiment 2.1.2. Effects of 2nd PCI after resolution of HP				
Experiment 2.1.3. Effects of 2nd & 3rd PCI prior to resolution of HP				

Task 1.0 (Months 1-6) Regulatory review and approval processing for studies involving animal subjects. The following animal protocols have been approved by the WRAIR IACUC: WRAIR IACUC Protocol # 12-PN-18S and 13-PN-30S. ACURO approval has been obtained for each of the study protocols. All regulatory review/approval requirements have been completed.

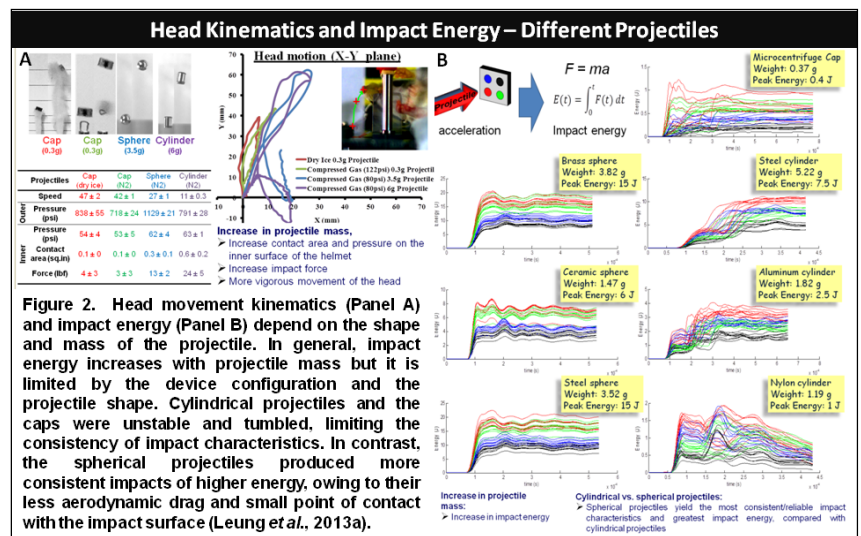
During this timeframe, several engineering components of the PCI model were refined to provide optimal injury parameters. The original PCI device used dry ice sublimation to build up pressure inside a microcentrifuge tube and trigger the release of a small projectile (i.e. the microcentrifuge cap) targeted to impact a helmet-protected rat head. However, we subsequently identified several limitations to the dry ice sublimation/microcentrifuge tube method and these limitations have been addressed by modifications made to (A) the PCI device and more recently to (B) the projectile. In addition, two pilot projects were conducted to determine (C) the optimal angle of PCI injury and (D) to establish a positive PCI control group. These modifications and results are summarized below:

(A) PCI Device: Started during the past year and completed during 1st QTR (FY13 Q1) of this proposal, the PCI device was modified to use compressed gas (i.e. nitrogen) instead of dry ice sublimation as the trigger mechanism for launching the projectile. In addition, a computer control interface was implemented to control the operating pressure (Figure 1). The primary advantage of using compressed gas vs. dry ice sublimation is that the mechanical forces used to induce the injury are far more controllable, reproducible and quantifiable. In

addition, the “pressure wave” generated by the release of compressed gas is of low magnitude and is not related to the input pressure. Thus, the “pressure wave” effect is minimal and can be more effectively controlled. **Moreover, the intensity of the force can be titrated to produce a wider spectrum of closed-head concussive injury severities for study.** A patent application was submitted for this iteration of the device in August 2012 (U.S. Provisional Application Serial No. 61/521,446).

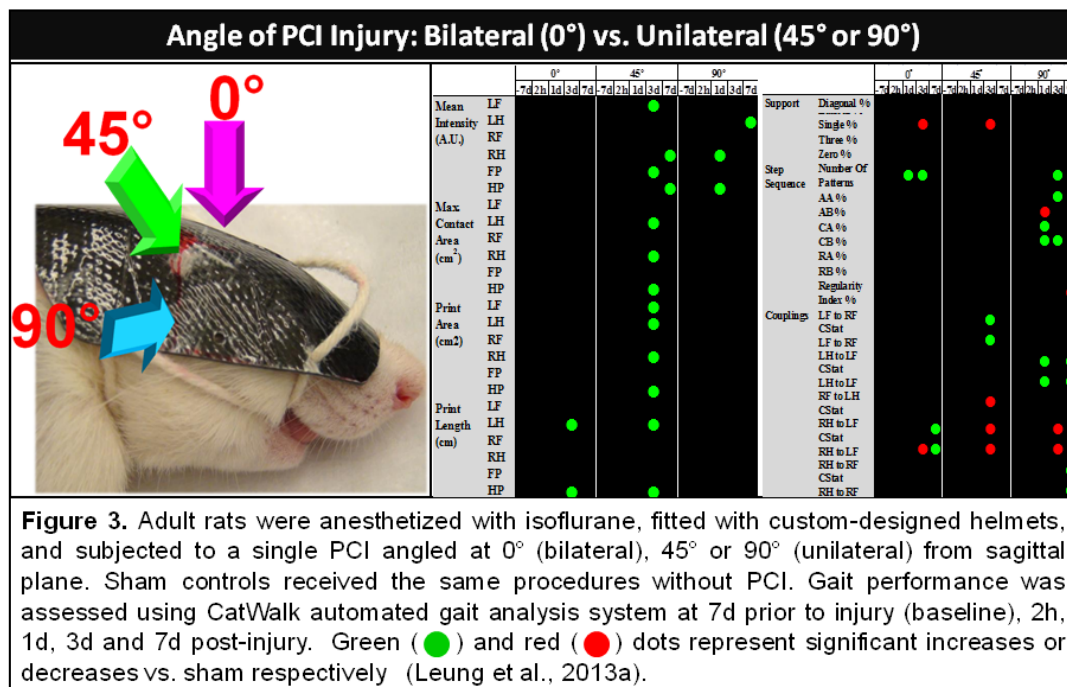


(B) PCI Projectile: In addition to intervals between repeated injuries; varying the intensity or severity of the mTBI insult is a critical factor to evaluate in preclinical mTBI studies (Fujito et al., 2012). In keeping with this, the modifications made to the PCI device also facilitate the use of small projectiles of different shapes/masses. Thus, during the FY13 Q1 of this project we collaborated with the Army Research Laboratory (ARL; Aberdeen MD) to test a number of small spherical (i.e. ball bearings) and cylindrical projectiles of different masses (ranging from 0.5 to 6g). **The steel ball bearings have produced the most desirable and consistent pressure distribution profile on the inner surface of the helmet while remaining within a range that meets the criteria for mTBI.**



(C) Angle of PCI Injury: In an initial pilot experiment, we assessed PCI-induced injuries that were angled (A) 0° from the sagittal plane (bilateral hit) or (B) either 45° or 90° from the sagittal plane (unilateral hits). CatWalk automated gait analysis (Noldus, The Netherlands) was used to detect gait abnormalities at 2h, 1, 3, 7 days post-injury. Results showed that unilateral PCI produced a greater degree of gait alterations compared to bilateral PCI demonstrated by alterations in 46 or 32 (out of 210) gait parameters following the 45° and 90° hits respectively. In contrast, only 18 gait parameters were significantly altered following the bilateral (0°) PCI injury. Figure 3 provides a summary of the significant gait alterations detected in the three groups at different time points. Significant increases in mean intensities of both front and hind paw prints were observed in rats

subjected to unilateral hits (45° and 90°) at 1, 3 or 7 days post-injury ($p < .05$ vs. sham control). ***Unilateral PCI angled at 45° produced the most robust gait abnormalities that are sustained under repeated testing conditions.***



SUMMARY OF ADVANCED PCI MODEL: Carbon/glass fiber composite material is used for helmet fabrication; (2) the microcentrifuge cap in the original model has been replaced by a steel sphere (3.52 g) as the projectile; (3) pressure used to launch the projectile is set at 80 psi; and (4) the impact location is set at a 45° angle targeting the temporoparietal region (right hemisphere). These advancements have been presented at the Society for Neurotrauma Symposium in Nashville TN (Leung et al., 2013a) and are described in greater detail in Leung et al. (2014). ***All aspects and components of the refined/advanced PCI model were approved in the current WRAIR IACUC Protocols 12-PN-18S and 13-PN-30S.***

PCI procedure (used for all tasks outlined below): The PCI injury apparatus consists of an elevated platform and a computer-controlled electro-pneumatic pressure release system used to launch a small projectile (3.52 g sphere) targeted at the rat's head. Following anesthetization with 5% isoflurane, a custom-designed helmet (Army Research Lab, Aberdeen Proving Ground, MD) is securely fastened onto the rat's head. Pressure sensor films (Fujifilm pre-scale pressure sensitive film) adhered to the inner and outer surfaces of the helmet are used to record the distribution and magnitude of pressure from the impact of the projectile. The anesthetized rat is placed on the elevated platform with its head positioned above an oval opening in the elevated platform such that the right hemisphere of the helmet-protected head is exposed to the projectile angled 45° from the sagittal plane. A computer program is used to trigger the targeted release of the projectile at the rat's head. Immediately following PCI injury, the helmet is removed and the rat is returned to its home cage. Sham control animals receive the same procedures except the projectile impact.

The original study design called for the inclusion of a pressure wave (PW) control group to control for the potential effects of the PCI pressure wave. However, in the advanced PCI system, the need for a "pressure wave" (PW) control group has been negated by the refinements made to advanced PCI system because the "pressure wave" generated by the release of compressed gas is minimal. As a substitute for the PW group, we have included a positive PCI control group in the experimental design when needed to confirm that the outcome measures are capable of detecting injury signals. For this purpose, animals were subjected to 4 PCI-

induced concussions (1 hour apart), representing a more severe concussion, yet remaining within the limits of the mTBI spectrum.

SOW 1 (Months 1-24): Fully characterize a “SINGLE” PCI head injury defining the acute temporal profile of histopathology, molecular (biomarkers/bioenergetics), neurobehavioral (motor/cognitive) dysfunction, and electrophysiological (EEG) changes following a single PCI.

Section II

Task 1.0 (Months 1-6): All regulatory review/approval requirements have been completed.

Task 1.1 (Months 1-12): Evaluate the regional and temporal profile of cellular changes following a single PCI injury. The effects of a single, lateral PCI on axonal injury using APP and CuAg staining are to be assessed at 6h, 24h, 72h, 7d, 14d and 28d post-PCI. The regional profile of the glial response to PCI injury are to be assessed at 6h, 24h, 72h, 7d and 14d post-injury using immunostaining markers for reactive astrocytes and activated microglia. The effects of PCI on BBB permeability will be examined at acute post-injury timepoints (6h, 24h and 72h) using biotin dextran amine (BDA; 3 kDa) and by IHC using antibodies for (1) Aquaporin 4 (AQ4) co-labeled with GFAP, and (2) tight junction and endothelial linkage proteins occluden, zonula occluden 1 (ZO-1), and claudin-5 (CI-5).

Experiment 1.1.1 Diffuse Axonal Injury (DAI): DAI is a hallmark pathologic feature of TBI and has been consistently detected across the spectrum of TBI severities, including mTBI. Our proposed study will focus on the expression of beta-amyloid precursor protein (APP) and amino cupric silver (CuAg) expression as markers for acute axonal injury. The effects of PCI on axonal injury using APP and CuAg staining will be assessed at 6h, 24h, 72h, 7d, 14d and 28d post-PCI. **LEAD INVESTIGATOR – Dr. Lai Yee Leung, STATUS: Completed. Final results were included in Y2 Annual Report.**

Experiment 1.1.2. Glial Response: We previously reported significant increases in hippocampal expression of GFAP (glial fibrillary acidic protein; a marker for reactive astrocytes) in the PCI model at 24h post-injury. In the proposed study, the glial response to PCI injury will be examined in different brain regions (cerebral cortex, hippocampus, corpus callosum, thalamus, striatum and cerebellum) at 6h, 24h, 72h, 7d and 14d post-injury using immunostaining markers for reactive astrocytes and activated microglia. **LEAD INVESTIGATOR – Dr. Lai Yee Leung, STATUS: Completed. Final results were presented in Y2 Annual Report and Y3Q1 Quarterly Report.**

Experiment 1.1.3. Blood-Brain Barrier (BBB) Permeability: The effects of PCI on BBB permeability will be examined at discrete post-injury time points (i.e. 6h, 24h and 72h) using biotin dextran amine (BDA; 3 kDa) to detect more subtle BBB disruption that may not be apparent using Evan's blue extravasation or serum albumin IgG methods. In addition, the involvement of astrocytes and/or tight junctions in the BBB breakdown process will be examined by IHC using antibodies for (1) Aquaporin 4 (AQ4) co-labeled with GFAP, and (2) tight junction and endothelial linkage proteins occluden, zonula occluden 1 (ZO-1), and claudin-5 (CI-5). **LEAD INVESTIGATOR – Dr. Lai Yee Leung; STATUS: Completed. Final results were included in Y2 Annual Report. Dr. Jenny Browning presented this research as a poster at the 2015 National Neurotrauma Symposium.**

Task 1.2 (Months 6-18): Evaluate the regional and temporal profile of molecular/bionergetic changes in brain tissue following a single PCI. **Exp. 1.2.1:** Changes in messenger ribonucleic acid (mRNA) levels will be evaluated following a single PCI injury in brain lysate by real-time polymerase chain reaction (PCR) with primers specific for known markers of cellular injury (i.e. GFAP, UCH-L1, Alpha-II spectrin, and APP). **Exp. 1.2.2:** Changes detected in mRNA expression will be correlated with changes in protein expression. **Exp. 1.2.3:** Changes in metabolic activity levels will be assessed using ultra-performance liquid chromatography (UPLC) measurements of adenosine triphosphate (ATP), adenosine diphosphate (ADP), creatine,

phosphocreatine and N-acetylaspartate (NAA) levels to establish a profile of metabolic vulnerability/recovery in the PCI model.

Experiment 1.2.1. Molecular Changes: Changes in messenger ribonucleic acid (mRNA) levels will be evaluated following a single PCI injury in brain lysate by real-time polymerase chain reaction (PCR) with primers specific for known markers of cellular injury (i.e. GFAP, UCH-L1, Alpha-II spectrin, and APP) at 2h, 6h, 24h, 72h, and 7d post-PCI in comparison to sham and PW controls. We will correlate changes in mRNA expression with changes in protein expression (Exp. 1.2.2) to determine the precise mechanism of injury (i.e. gene regulation vs. protein modification). **LEAD INVESTIGATOR – Dr. Casandra Cartagena; STATUS: Completed. Final results were included in Y2 Annual Report and Y3 Q1 Quarterly Report.**

Experiment 1.2.2. Protein Biomarkers: Changes in protein abundance for known markers of cellular injury (i.e. GFAP and its BDPs, UCH-L1, SBDPs, and c-APP) will be evaluated following a single PCI injury in brain tissue, cerebral spinal fluid (CSF) and serum by Western blot or enzyme-linked immunosorbent assays (ELISAs) following a single PCI injury at 2h, 6h, 24h, 72h, and 7d post-injury in comparison to sham and PW controls. **LEAD INVESTIGATOR – Dr. Angela Boutte; STATUS: Completed. Final results were included in Y2 Annual Report and Y3 Q1 Quarterly Report. Dr. Boutte presented this research as a poster at the 2015 National Neurotrauma Symposium.**

Experiment 1.2.3. Bioenergetic Profile: Changes in metabolic activity levels will be assessed following a single PCI injury using the electromagnetic tissue fixation method to prepare brains for ultra-performance liquid chromatography (UPLC) measurements of adenosine triphosphate (ATP), adenosine diphosphate (ADP), creatine, phosphocreatine and N-acetylaspartate (NAA) levels to establish a profile of metabolic vulnerability/recovery in the PCI model. **LEAD INVESTIGATOR – Dr. Ying Deng-Bryant; STATUS: Completed. Final results were included in Y2 Annual Report.**

Experiment 1.2.4. microRNA Biomarker Profile (serum): microRNA Profiling as a novel biomarker for mTBI was added to this study in Y2. The miRNA profile in serum will be evaluated following a single PCI injury will be evaluated at 4h, 24h, 3d, and 7d. **LEAD INVESTIGATOR – CPT David Johnson; STATUS: Completed. Final results were included in Y2 Annual Report and the Y3 Q2 Quarterly Report and are summarized below. CPT Johnson presented this research as a poster at the 2015 National Neurotrauma Symposium and was invited to give an oral presentation at the 2015 Military Health System Research Symposium.**

Investigation of microRNAs (miRNAs) as putative biological indicators of injury has been examined in many disease states. MiRNAs regulate many cellular processes through translational repression or degradation. In this study we used the projectile concussive impact (PCI) model and microarray platform to examine whether miRNAs may serve as indicators of mild traumatic brain injury (mTBI). This injury model represents a mild clinically relevant injury. Briefly, PCI injury induction is a non-invasive, closed-head blunt impact to the right temporoparietal region. Sham animals received equivalent anesthesia without impact. Serum was collected from rodents at 4h, 1d, 3d, or 7d following injury and miRNA dysregulation was measured in rodents. MicroRNA arrays were performed using Taqman megaplex reverse transcription and pre-amplification kits. Each animal was run as a singlet independent array (n=10/group). We limited our reporting to miRNAs with p value <0.05 and a two-fold or greater change. Given these criteria for significance, we found two upregulated and two downregulated miRNAs at the 4 hr time point, four miRNAs that were upregulated at 1d, and four miRNAs that were upregulated at 3d. No changes in regulation of miRNAs at 7d were observed. **(Appendix B Figures 1 and 2). The serum miRNAs changed following PCI will be further studied to determine their usefulness as both diagnostic and prognostic indicators of mTBI.**

Task 1.3 (Months 1-18). Evaluate the neurobehavioral (motor, cognitive, and affective) profile following PCI injury. The goal of these experiments is to establish a comprehensive longitudinal neurobehavioral assessment of a single PCI injury on motor, cognitive, and affective (i.e. depression/learned helplessness) abnormalities. The key outcome metrics effects will be the degree of functional deficits on (1) a computer-assisted gait task, (2) a rotarod task, (3) a Morris water maze task, (4) a novel object recognition (NOR) task, and (5) a forced swim task. Groups will consist of sham, PW controls, and PCI (n=15/group) and separate groups of animals will be assessed for functional impairment on the respective tasks at both acute (< 3 days) and chronic (7-28 days) time points as outlined in tables provided in the SOW. **LEAD INVESTIGATORS – Dr. Ying Deng-Bryant, CPT Andrea Mountney; STATUS: Completed. Final results were included in Y2 Annual Report and Y3 Q1 Quarterly Report.**

Task 1.4 (Months 12-24). Evaluate quantitative electrophysiological (qEEG) profile of PCI-induced abnormalities in brain wave patterns: EEG power spectrum analysis was used to examine EEG power shift and altered EEG coherence following PCI, through continuous EEG recording out to 72h post-PCI, followed by a 2-h recording on post-injury Days 5, 7, and 14. Experimental groups consisted of sham, PW controls, and PCI (n=15/group; N=45). **LEAD INVESTIGATOR – Dr. Xi-Chun May Lu; STATUS: Completed. Final results were included in Y2 Annual Report and Y3 Q1 Quarterly Report. Dr. Lu presented this research as a poster at the 2015 National Neurotrauma Symposium.**

SOW 1 Summary and Conclusions: Table 1 (Appendix B) provides a “heat-map” profile of our most current results for single and repeated (4 hits spaced 1 hour apart) concussion in the PCI model. Results for each Task (i.e. Neuropathology, Molecular and Functional Outcomes) are summarized below:

Neuropathological Results: A single concussion produced significant bilateral increases in accumulation of β -APP indicative of axonal damage that peaked at 6h post-injury and were resolved by 72h. Corresponding hippocampal GFAP levels were slightly upregulated at 6h following and were significantly higher than sham at 24h in both hemispheres, indicative of progressive astrocyte activation. Significant microglial activation, indicated by Iba-1, was evident at 6h and 72h in the hippocampus following a single concussion (vs. sham) that was resolved at 7 days post-injury. Additionally, a significant increase in neuronal cell death (flourojade) was detected at 24h post-injury. Of these measures, β -APP, Iba-1 and flourojade were significantly higher following repeated vs. single concussion for at least one post-injury time point (**Appendix B Figure 3**).

Molecular Results: Western blot results showed no changes in brain tissue protein levels for GFAP, UCH-L1, SBPD, β -APP, Tau or p-Tau following a single concussion. However, Tau and p-Tau were significantly elevated in the hippocampus at 72h post-injury following repeated concussion. CSF biomarker results showed significant increases in GFAP and UCH-L1 at 1h post-injury and Tau at 24h post-injury following single and repeated concussion. Additionally, inflammatory cytokines were significantly upregulated in CSF at 1h and 24h post-injury following repeated PCI. Similar results were detected in serum biomarkers with inflammatory miRNAs (4h, 24h, and 3d) (**Appendix B Figures 1-2**) and inflammatory cytokines (1h) (**Appendix B Figure 4**) showing significant increases following a single concussion and GFAP showing a significant increase at 1h post-injury following repeated PCI. Of these results, miRNA measures appear to provide the most promising therapeutic target in serum) whereas Tau and inflammatory cytokines may provide additional targets in CSF

Functional Outcomes: PCI produces acute (\leq 4h) abnormalities in righting reflex (not shown), NSSR and BBB scores following both single and repeated concussion that are resolved by 24h post-injury. ***Decrements in rotarod and MWM performance were detected only following repeated concussion.*** However, significant alterations in sensorimotor (gait) activity were detected on the CatWalk following a single concussion out to 72h post-injury (and again at 1 month post) that were significantly higher following repeat concussion (**Appendix B Figure 5**). Overall, these results indicate that, while the righting reflex, BBB and NSSR scores provide a useful inclusion/exclusion criteria matrix, the CatWalk provides the most useful metric for evaluating putative therapeutic effects during the acute post-injury phase.

SOW 2 (Months 24 - 48): Evaluate the cumulative effects of “REPEATED” PCI longitudinally across outcome metrics defined in SOW 1. Recent studies have implied that there is a correlation between sustaining repetitive concussions and experiencing worse outcomes, and it has been suggested that repetitive concussions can lead to the development of chronic pathological and psychological changes in the concussed subject. However, very little is actually known or understood about repetitive mTBI events and what factors are associated with the debilitating outcomes. Currently it is not known what changes are taking place in the brain with a concussion, how long the changes last and what happens if a second (or third, fourth...etc) concussion is experienced during the healing period. Therefore, the goal of this SOW is to determine the effects associated with repetitive concussive injuries.

Task 2.1. Determine the effect of “REPEATED” PCI injuries compared to a “SINGLE” PCI injury based on the healing profile. The experimental design and specific time points for repeated PCI injuries for SOW 2 will be based on the optimal outcome metrics and time points identified in SOW 1. More specifically, the data generated in SOW 1 will fully characterize the injury profile in the brain after a single PCI injury. Based on this data, a “healing profile” will be developed that demonstrates the time at which the brain has essentially returned to normal based on the associated neuropathological, molecular and neurobehavioral changes. We will evaluate the effects of repeated PCI injuries occurring either before or after resolution of the healing profile. For example, if the data from a single PCI injury in SOW 1 demonstrates that the healing profile has resolved by 72h post-PCI, then separate groups of animals will be subjected to additional PCI injuries at either (A) 24h and/or 48h post-PCI (during the healing period) or (B) 24h after resolution of the healing profile (i.e. 4 days post-PCI).

Task 2.1.1 Regional and temporal profile of neuropathological alterations following repeated concussion: Phase I (SOW I) results indicates that the healing profiles (i.e. when significant alterations induced by the injury are no longer detectable) for axonal injury and BBB permeability are ≤ 24 h whereas the healing profile for the neuroinflammatory response (GFAP and Iba-1) is approximately 72h. Based on those results, *Phase II animals will be exposed to either 2 or 3 PCIs spaced 24h apart (during the healing period) or 2 PCIs spaced 7 days apart (after resolution of the healing profile).* As before, separate groups of animals will be used for BBB immunostaining (AQ4, ZO-1 and CL-5) due to the need to use fresh frozen tissue. For all markers, animals will be euthanized at 24h, 72h and 7d following the last PCI (additional post-injury time points may be added as indicated by the results). **LEAD INVESTIGATOR – Dr. Lai Yee Leung; STATUS: All brain samples for Experiment 2.1.1 regarding BBB immunostaining have been collected and are currently being processed. There are no additional results for BBB immunostaining to report at this time.**

Based on the phase-I neuropathological results, we expanded the neuroinflammatory study by characterizing microglial phenotypes during the peak activation time points following repeat injury. **LEAD INVESTIGATOR – Dr. Sindhu Madathil; STATUS: Studies are ongoing. Results determined to date are summarized below.**

Experiment 2.1.1.1. Microglial Phenotype: To understand the microglial phenotype changes following concussive brain injury, single and repeat PCI (4 hits; 1h interval) injuries were conducted. Two endpoints (6h and 72h) were selected based on earlier experiments that showed increased Iba-1 (microglial marker) staining following PCI in rats. Following intra-cardiac perfusion, brains were harvested and processed for immunostaining. Coronal sections (30 μ m) were immunostained with MHC-II/Iba-1 (M-1 phenotype) or CD163/Iba-1 (M-2 phenotype) antibodies. M-1 (co-labelled for MHC-II and Iba-1) and M-2 (co-labelled for CD163 and Iba-1) microglia in different brain regions (cortex, hippocampus and striatum) were counted manually at 20X magnification. M-1 or pro-inflammatory phenotype was mostly located in the cortex whereas the anti-inflammatory M-2 phenotype was predominate in cortex, striatum and hippocampus. Repeat PCI

increased M-1 type expression bilaterally compared to single PCI, and concomitantly downregulated M-2 type microglia at both time points studied. At 6h, following repeat PCI, the increase in M-1 signal was 39% and 62% respectively for contralateral and ipsilateral cortex, whereas much higher increases in M-1 expression were detected at 72h post-injury (647% and 1500% respectively for contra and ipsilateral cortex). M-2 microglial counts were decreased by 26% and 14% at 6h respectively in contralateral and ipsilateral cortex. At 72h, the decline in M-2 phenotype was more pronounced with 50% and 30% respectively for contralateral and ipsilateral cortex. Moreover, repeat PCI increased M-1/M-2 ratio bilaterally compared to single PCI (89% increase at 6h compared to single PCI) indicating a shift towards inflammatory phenotype (**Appendix B Figures 6-7**). **These findings indicate microglial polarization following concussive brain injury. Following multiple concussions, the microglial phenotype showed a shift towards proinflammatory M-1 type, a response that may serve as a novel therapeutic target for TBI.**

Task 2.1.2. Regional and temporal profile of molecular changes following single and repeated PCI injury:

Experiment 2.1.2.1. Protein Biomarkers (brain tissue): UCHL1 has been eliminated as a target and replaced with the more relevant tau protein targets. Phase I studies showed acute (4h) transient reductions in GFAP and GFAPbdp levels following a single PCI in the cortex and hippocampus (injured hemisphere). In addition evidence of increased levels of total tau and tau phosphorylation levels at 72h following repeated PCI (i.e. 4 hits spaced 1h apart), but not single PCI. Phase II research will look at 24 and 7 day intervals and at later time points post injury (7 days) to determine whether significant alterations in total tau and tau phosphorylation levels are evident under these conditions. In addition we are currently validating a new method of detecting APP processing pathological fragments (amyloid forms) and will evaluate whether these are altered following PCI in CSF and serum. **LEAD INVESTIGATOR – Dr. Casandra Cartagena; STATUS: Complete. Results are summarized below. Dr. Cartagena presented this research as a poster at the 2015 Society for Neuroscience Annual Meeting.**

Repeat PCI injuries (4 injuries) were conducted at 1h, 24h or 7d inter-injury intervals. Experimental endpoints included 3d after the last injury (all injury cohorts) as well as 4h, 24h and 7d post-rPCI (1h inter-injury intervals only). Following 4 PCI injuries spaced 1h apart, significant increases in tau (33%, **Appendix B Figure 8**) and phosphorylated tau (28%, **Appendix B Figure 9**) were detected in the HC at 3d post-injury that were normalized by 7d post-injury. Tau phosphorylation decreased 24h post-injury following rPCI with a 1h interval in CX (**Appendix B Figure 8**). No other changes were found in the CX. No changes were found in APP or APP beta cleavage at 3d or 7d (data not shown). Increased GFAP (138%, **Appendix B Figure 10**) and SBDP (41%, **Appendix B Figure 11**) were seen in CX but not HC 3d following rPCI with 1hr interval. Because significant changes were seen in multiple pathology markers 3d post rPCI additional intervals between impact concussions were evaluated at this time point. Following 4 PCI injuries spaced 24h apart, no changes were seen in tau phosphorylation, APP or APP beta cleavage at 3d post injury. However increased GFAP was detected in both CX (207%) and HC (44%, **Appendix B Figure 10**) and increased SBDP (43%, **Appendix B Figure 11**) in CX at 3d. No significant changes were detected in any of these markers following 4 PCI injuries spaced 7d apart. Notably, the 24h interval between impact concussions produced substantially greater GFAP upregulation vs. the 1h interval, an affect that was observed in both CX and HC regions. In addition, 2 PCI injuries 24h apart were evaluated 3d post-injury and showed no significant changes. Significant changes in pathological markers (tau and tau phosphorylation in HC at 4xPCI 3d 1h interval, GFAP and SBDP in CX at 4xPCI 3d 1h and 24 hr intervals) were evaluated for correlation with times to regain the righting reflex (**Appendix B Figure 12**). While Tau and SBDP showed some correlation of righting reflex measures, the strongest correlation was to GFAP. In addition, only GFAP showed positive correlation to the PSI transferred to the inside of the helmet (**Appendix B Figure 13**). **Overall, these results indicate that tau and tau phosphorylation are more affected following rapid repeat injuries; whereas GFAP changes are more**

prevalent with longer inter-injury intervals and correlate better with other injury metrics, and thus may prove more valuable for evaluating therapeutic interventions.

Experiment 2.1.2.2. Biomarkers (CSF and serum): Phase I studies showed significant increases in GFAP in CSF 1h following a single PCI. In Phase II studies, animals will be exposed to either 2 or 3 PCIs spaced 24h apart or 2 hits spaced 7 days apart. **LEAD INVESTIGATOR – Dr. Angela Boutte; STATUS: Studies are ongoing. Results determined to date are summarized below. Dr. Boutte presented this research as a poster at the 2015 National Neurotrauma Symposium.**

Initial studies determined the concentrations and fold-change values of GFAP, Tau, and UCH-L1 in the CSF collected at 1h after either a single (1X) or an iterative series of repeated (2-4X) PCIs. In addition, the effects upon righting reflex, sensorimotor deficits defined by the revised composite neurological severity scale were determined in order to establish biomarker – neurological deficit relationships. RR time and NSS-R scores increased in all injured groups, yet peaked after 2XPCI (**Appendix B Figure 14A-B**). CSF GFAP was elevated in all PCI groups and showed the greatest fold-change after 1XPCI. In contrast, Tau was greatest in CSF after 4XPCI. UCH-L1 levels were elevated in all repeated injuries and greatest after 2XPCI (**Appendix B Figure 15**). Tau, alone, correlated to RR time after 1XPCI, whereas all three biomarkers aligned with RR time after 2-4XPCI (**Appendix B Figure 16**). Only UCH-L1 was associated with NSS-R after 2-4XPCI (**Appendix B Figure 17**). **Thus, the PCI model effectively recapitulates several characteristic features expected in mTBI/concussion, such as increased latency to regain consciousness (RR time), sensorimotor deficits (composite and individual NSS-R), and elevation of putative TBI biomarkers within the CSF.** This model potentially foreshadows differences in glial vs. neuronal susceptibility as a consequence of concussion frequency during the acute time-frame and infers that tau and UCH-L1 are useful in establishing correlates to RR and NSS-R deficits, specifically due to repeated insults. **Thus, single and repeated PCI led to unique biomarker and neurological deficit profiles, such that a repeated incidence may not necessarily be defined as a more robust version of a single injury.** Testing for GFAP, Tau, and UCH-L1 using novel ultrasensitive assays has continued for single and repeat PCI cohorts collected at 1 and 24h after injury. In addition, CSF was collected at 1h and 24h after either 1XPCI or 4X PCI with the 1h interval. To date, analyses of biomarkers in CSF and serum, or plasma, is ongoing. These findings indicate that CSF GFAP is a biomarker of 1X and 4X PCI, detectable at 1h post injury (**Appendix B Figure 18-19**). At 24h, CSF GFAP was still significant. However, the response in tau at this time point was much more robust. Expanding the interval between injuries to 24h indicated that tau remained elevated, while UCH-L1 was decreased in the CSF of 4XPCI cohorts (**Appendix B Figure 20**). **Therefore, Tau may become a lead biomarker for biofluids in the PCI model.**

Recent research led us to expand our investigation of tissue pathology markers and biomarkers to include Cathepsin B. The purpose of this preliminary study was to determine if brain cathepsin B is up-regulated following repeated projectile concussive impact (rPCI) in rodent models of TBI. LEAD INVESTIGATOR – Dr. Angela Boutte; STATUS: Studies are ongoing. Results determined to date were reported in Y3 Q2 report and are summarized below. Dr. Boutte presented this research as a poster at the 2015 National Neurotrauma Symposium.

Comprehensive analysis of key mediators involved in traumatic brain injury (TBI) is tantamount to understanding mechanisms involved in injury progression. Cathepsin B is a cysteine protease implicated in several neurodegeneration and TBI models, such as controlled cortical impact. Repeated (r)PCI was conducted once daily for 4 consecutive days (d) where control groups received anesthesia alone. Righting-reflex (RR) was determined immediately after injury. Select PCI brain tissue regions were collected 1d after the last concussion. Both pro- (~37-43kDa) and mature (~20-25kDa) Cathepsin B protein levels were determined by western blotting and densitometry (mean+/-SEM arbitrary units (AU)). Enzymatic activity was determined by generation of amino-methyl coumarin (AMC) in a fluorescent micro-plate assay. Comparisons between injured and control groups are discussed (2-tailed, t-Test, $p \leq 0.05$) and correlative analysis is indicated (1-way,

Pearson r). Pro-cathepsin B was increased following rPCI to (1.7+/-0.5 AU) in the prefrontal cortex, but not detectable in sham/anesthesia controls. In this brain region, the mature form was nearly 7-fold greater after rPCI (14.2+/-3.6 AU) compared to controls (2.2+/-1.5 AU) and proteolytic activity was marginally increased. Surprisingly, proteolytic activity in the cerebellum increased by nearly 3-fold after rPCI (3.0+/-0.6 μ moles) compared to anesthesia alone (1.4+/-0.2 μ moles), and was positively associated with RR ($r = +0.65$, $p=0.12$). Conversely, decreased activity in this brain region was negatively correlated with RR ($r = -0.98$, $p=0.008$) among anesthesia controls. Overall, Cathepsin B was upregulated in mild injury models (rPCI). Furthermore, activity correlated to the inability to regain consciousness after concussion. ***These findings suggest that brain cathepsin B has a role in multiple TBI models and is linked to neurological deficits.***

Experiment 2.1.2.3. Bioenergetic Profile (brain tissue): Phase 1 studies showed significant alterations in metabolic activity levels following a single PCI that were evident from 30 min. to 6h post-injury (peak level) that were primarily resolved by 24h post-injury. For our initial Phase II experiments, rats will be exposed to 2 PCIs spaced 6h apart, 24h apart and 7 days apart. An additional group of animals will be exposed to 3 PCIs spaced 6h apart. All animals will be euthanized at 2h following the last PCI. **LEAD INVESTIGATOR – Dr. Ying Deng-Bryant; STATUS: Completed. Results were reported in Y3, Q2 and are summarized below. Dr. Bryant was invited to give oral presentations on this research project at the 2015 National Neurotrauma Symposium, the 2015 Military Health System Research Symposium and the 2015 Society for Neuroscience Annual Meeting.**

Repeated PCI injuries (2 injuries total) were conducted spaced 6h, 24h, or 7 days apart (**Appendix B Figure 21**). All animals were euthanized at 2h following the last PCI. Ipsilateral frontal cortex where the primary impact occurred was dissected. Metabolites from brain tissues together with CSF were extracted for metabolomic analysis. Mass spectroscopy-based metabolomics was performed to determine biochemical signatures in the biological samples, followed by statistical and pathways analysis to interpret the data (**Appendix B Figure 21**). The results revealed a total of 447 and 404 biochemicals in brain tissue and CSF respectively. Repeated (2 \times) concussions induce over 20% changes in the brain with 6 and 24 hour interval (**Appendix B Figure 22**). At 7 days interval, it is slightly lower at 15%. More importantly, CSF responds to repeated concussions differently from the brain. Only repeated concussion group with 6 hour interval induced a high percentage of change (in metabolites) in the CSF, suggesting more injured cells following concussions with shorter intervals, which can lead to higher dysregulation of metabolites in the CSF (**Appendix B Figure 22**). ***Overall, these results suggest a differential threshold of the brain vs. the CSF in response to concussion intervals with regards to the number of biochemical alterations.*** An additional group of animals were then exposed to 3 \times PCIs spaced 6h apart and compared with groups that received 1 or 2 \times PCIs. The results show an increased risk of disrupted metabolic homeostasis associated with repeated concussion versus a single concussion. More specifically, 3 \times PCIs resulted in increased levels of glycogen degradation, evidenced of enhanced oxidative stress, and higher tissue levels of polyamine metabolites (**Appendix B Figure 23**). ***Overall, these findings demonstrate a differential metabolomic profile sensitive to the number of concussion as evidenced by the incremental increases in levels of biochemical alterations. Further analysis will focus on the specificity of selected biochemical signatures that warrant analysis for their potential as concussion biomarkers.***

Experiment 2.1.2.4. microRNA Biomarker Profile (serum): microRNA Profiling as a novel biomarker for mTBI was added to this study in Y2. Phase I studies showed significant alterations serum miRNA levels following a single PCI evident at 4h and 24h post-injury. Phase II experiments, rats will be exposed to 2 PCIs spaced 24h apart and 7 days apart. An additional group of animals will be exposed to 3 PCIs spaced 6h apart. **LEAD INVESTIGATORS – CPT David Johnson and Dr. Bernard Wilfred; STATUS: Samples have been collected and are being analyzed. There are no additional results to report at this time.**

Task 2.1.3. Evaluate the neurobehavioral (motor, cognitive, and affective) profile following repeated PCI injury.

Experiment 2.1.3.1. Sensorimotor Measures: In Phase I experiments, evidence of sensorimotor impairment was quantified using a number of outcome metrics including the NSSR (neurological severity scale revised) task, gaitwalk and rotarod tasks. Overall, the results indicated that a single PCI produced some acute alterations that were evident up to 4h post-injury. Studies to investigate temporal profile of behavioral abnormalities following a second PCI are currently underway. The effects of repeated concussion on gait abnormalities (CatWalk) and vestibular dysfunction (rotarod) with injuries a single PCI (one time point), spaced 24h apart (24PCI, 2x24h) and spaced 7 days (7dPCI, 2x7d) apart will be examined. **LEAD INVESTIGATOR – CPT Andrea Mountney; STATUS: Studies of gait analysis are complete. Results are summarized below. Ongoing studies will determine whether endpoint histological analysis of these animals correlates with sensorimotor findings.**

The aim of this study was to assess the effects of multiple concussions occurring at different intervals on sensorimotor function. For this purpose, rats received two consecutive projectile concussive impacts (2×PCI) at 24h or 7d intervals; each paired with a procedure-matched sham controls (anesthesia only). Sensorimotor abnormalities were assessed using the CatWalk gait analysis system, the neurological severity score-revised (NSS-R) and rotarod. PCI injury produced subtle, yet significant differences in fine sensorimotor parameters such as stance, swing speed, and step cycle with no overt changes in coordination (regularity index). All PCI-injured animals showed decreased cadence, increased stance, and decreased swing-speeds. Deficits were equally distributed between the four limbs and appeared acutely, with progressive resolution over 2-7 days post-injury. The majority of changes were detected at 2h post-injury: sPCI(25) rPCI24H(8) and rPCI7D(16). However, these deficits were transient. By 24h post-injury, sPCI, rPCI24 and rPCI7D showed 80%, 63%, and 100% reductions in abnormal gait parameters, respectively. **Critically, all PCI-injured animals exhibited a bi-phasic profile showing delayed reemergence of gait abnormalities.** Specifically, at 28d the rPCI24H group showed the greatest number of gait alterations (19) compared to sPCI (6) and rPCI7D (12) (**Appendix B Figure 24**). **All animals subjected to repeated concussion at 24h intervals displayed significant changes in interlimb support, adopting an overly reliant tri-limb walking pattern.** Additionally the rPCI24H rats showed significant deficits in motor learning capacity (rotarod task) and highest NSSR scores, indicative of chronic neurologic damage, compared to all other groups (**Appendix B Figure 25-26**). **Overall, we found that repeated concussion significantly altered sensorimotor function with the greatest decrements evident with two concussions occurring within 24h.** Additional studies are ongoing to evaluate the effects of different intervals between hits (i.e. 48 to 72h) and to identify potential correlations between sensorimotor and molecular changes (histology). However, **these initial results provide further validation for usefulness of the PCI model as a research tool to better understand the time frame during which the injured brain is more vulnerable to repeat concussions and ultimately, to evaluate the therapeutic efficacy of promising treatment strategies.**

Experiment 2.1.3.2. Cognitive Measures: Phase I experiments failed to show any evidence of cognitive dysfunction in the MWM or NOR tasks following a single PCI. However, additional studies evaluating the effects of repeated concussion (4 PCIs spaced 1h apart) did demonstrate spatial learning and working memory deficits at 1 month and 6 months post-injury. Our initial Phase I studies evaluated more acute working memory deficits following repeated PCI (4x; 1h apart). We are also currently evaluating the effects of repeated PCI (4x; 1h apart) in the NOR in order to determine which task would be more sensitive to repeated concussion. **LEAD INVESTIGATOR – Dr. Ying Deng-Bryant; STATUS: Acute MWM studies of repeated PCI have been completed. Results were included in the Y3 Q1 Quarterly Report and summarized below.**

The sham control group received a single anesthesia (4% isoflurane), whereas the PCI positive control group received four PCIs at 1 hour intervals. All rats were subjected to two pairs of working memory task on each testing day (total 4 trials per day). Rats were tested in a circular pool (75cm deep and 175cm in diameter) filled

with clear water. The starting position and platform location are the same within the trial pair, but different from other trial pairs. **Overall, the results indicated a significant effect on the working memory task between the sSham vs. the rPCI groups on 2 and 3 days post-injury (DPI) ($\dagger p < 0.05$) (Appendix B Figure 27).** Two-way repeated measures ANOVA was significant for trial effect on 3 DPI as both groups located the platform significantly faster in trial 2 than trial 1 in the paired trials ($*p < 0.05$). No difference was detected on 7 and 14 DPI. Significant between group effect (sSham vs. rPCI) were detected on 21 DPI ($\dagger p < 0.05$). Two-way repeated measures ANOVA was significant for trial effect on 21 DPI as both groups located the platform significantly faster in trial 2 than trial 1 in the paired trials ($*p < 0.05$). Two-way repeated measures ANOVA was significant for group \times trial interaction ($\dagger p < 0.05$) on 28 DPI as the rPCI group performed much better than sSham over the two paired trials.

Experiment 2.1.3.3. Affective Behavioral Measures: In Phase II negative affect was evaluated following repeated PCI (4x; 1h apart). Three tests were carried out to investigate negative affect: the air-puff induced negative vocalization test (acute), the elevated plus maze test (acute and 1 month) and the forced swim test (acute and 1 month). **LEAD INVESTIGATOR – Dr. Jenny Browning; STATUS: Studies are ongoing. Results determined to date are summarized below.**

Animals that received 4 concussive injuries produced more negative vocalizations in response to the same mild negative air-puff stimulus compared to sham animals. In addition, the injured animals vocalized for a longer duration in response to the air-puff procedure. There was also a strong trend toward a lower threshold for vocalization in the injured animals ($p = .05$) (**Appendix B Figure 28**). Additionally, the injured animals showed deficits in investigation and/or locomotion 3 days following injury in the EPM. The distance travelled and velocity were both lower in injured animals. The number of closed arm entries was also lower. Low levels of movement may indicate a deficit in locomotor behavior or a lower level of investigation due to increased anxiety. Locomotor deficits have been seen in the current model, however these are minor deficits in the gait of the animal and may not significantly contribute to lack of investigation of the maze (**Appendix B Figure 29**). The FST yielded no significant results, however the injured animals did show a trend toward increased mobility and decreased immobility when tested 7 days post-injury. If significant, these results would be in opposition to our original hypothesis, as lower immobility is thought to be indicative of a less depressive phenotype and our hypothesis was that the injured would show a more depressive phenotype (**Appendix B Figure 30**). **Overall, measures of negative affect were most apparent when evaluated by the air-puff induced negative vocalization test.** Negative vocalizations are an indication of general negative affect and do not indicate a specific type of negative state (eg sadness, anxiety, fear). Therefore, **the increase in negative affect observed in the present study could result from pain, anxiety, fear, or depressive states.** Further studies are underway using more sensitive testing paradigms for depressive states and anxiety to determine which states contribute to the negative affect revealed by the air-puff vocalization test. Identification of paradigms sensitive to our concussion model will allow us to test the ability of novel therapies to treat negative affect resulting from concussion.

Task 2.1.4. EEG Analysis: Investigation of EEG functional changes in the repeated PCI model will focus on the magnitude of EEG slowing and persistency of the abnormality. The frequency of repeated hits and the time interval between each hit for this study will be determined based on the outcomes yielded from ongoing behavioral and molecular studies of repeated PCI. **LEAD INVESTIGATOR – Dr. Xi-Chun May Lu; STATUS: Completed the study of EEG functional changes following repeated PCI using the 4xPCI with a 1h interval protocol. Results are summarized below. The study of other inter-injury intervals is ongoing.**

All animals received continuous EEG recording for 14 days immediately after the injury. qEEG power spectral analysis were performed *offline* from each rat at 12, 24, 48, 72 h, 7, and 14 days post-PCI or sham procedure. The results showed that repeated PCI injury caused EEG slowing during the initial 24-h post injury period in the ipsilateral hemisphere, evident by the significant increases in EEG delta power at 12 and 24 h post injury (p

< 0.05 vs sham at both time points). Similar trends in EEG slowing, albeit to a lesser degree, were also measured between 48 and 72 h post PCI ($p > 0.05$). Normal EEG activities were restored by the 7th day post injury and remained stable thereafter (**Appendix B Figures 31-32**). **Overall, the time-course profile of EEG slowing after repeated PCI appeared to be very similar to that following a single PCI as we reported previously, namely, the injury-related EEG slowing was restricted to the acute phase of brain injury. Thus, EEG function appears to recover spontaneously over time even after the repeated mild concussion.**

Section III

No Issues

Section IV

All tasks are progressing on schedule.

KEY RESEARCH ACCOMPLISHMENTS

Year 1 Accomplishments

1. IACUC and ACURO approval completed for two active protocols (in vivo and molecular).
2. Completion of Advanced PCI Model and Parameters to include PCI Device (driven by compressed gas vs. dry ice sublimation); completion of helmet material and design testing; PCI projectile; angle/location of PCI injury on the rat head.
3. Completion of acute (6h – 7 days post-injury) histopathological studies of a single PCI. Analysis of chronic post-injury time points is in process.
4. Completed collection of all histopathological samples for evaluating blood brain barrier (BBB) permeability following a single PCI. Brain tissues are being processed and analysis is targeted for completion in the first Quarter of Year 2.
5. Completed all tissue collections for mRNA molecular and protein biomarker changes. Analysis of the effects of PCI on GFAP and GFAP breakdown products has been completed. Analysis of additional markers is ongoing.
6. Completed sample collection for changes in metabolic activity levels. Primary (2h) samples are currently being processed via contractual agreement by Metabolon for global analysis of over 4,000 metabolites.
7. Completed acute post-injury assessment of motor (i.e. gaitwalk) abnormalities following a single PCI. Chronic evaluations are ongoing.
8. Completed acute post-injury assessment of cognitive (MWM) function following a single PCI. Additional animals are currently being tested at chronic time points.
9. Added righting reflex measures to the neurobehavioral outcome parameters and reported results confirming the validity of the PCI model as a model of closed-head concussive mild TBI.

Year 2 Accomplishments

1. Completed longitudinal (6h – 28 days post-injury) histopathological analysis of diffuse axonal injury and glial activation following a single PCI and repeated (4x) PCI.
2. Completed histopathological evaluation of blood brain barrier (BBB) permeability following a single PCI and repeated (4x) PCI.
3. Completed all tissue processing and analyses for mRNA molecular and protein biomarker changes and repeated (up to 4x) PCI.

4. Completed additional evaluations of phosphorylated Tau and total Tau following a single and repeated (4x) PCI.
5. Completed additional evaluation of novel microRNA biomarker analysis in serum following a single PCI.
6. Completed metabolomics analysis of all tissue samples and identified post-injury metabolic profile following a single PCI and repeated (4x) PCI.
7. Completed acute and chronic post-injury assessment of neurological (i.e. righting reflex and NSSR score) dysfunction following a single PCI and repeated (up to 4x) PCI.
8. Completed chronic post-injury assessment of cognitive (MWM) function following repeated (4x) PCI.
9. Completed acute EEG measurements of brain wave activity following a single PCI.

Year 3 Accomplishments

1. ***Initiated experiments to determine the effects of rPCI delivered pre- and post- resolution of the healing profile on BBB permeability.***
2. ***Added investigations to characterize microglial phenotypes following repeat injury paradigms; preliminary analysis demonstrates differential microglial activation following sPCI and rPCI.***
3. ***Completed evaluation of protein biomarkers in brain tissue including Tau, pTau, GFAP and SBDP following (4x) PCI induced at different inter injury intervals.***
4. ***Initiated investigations of CSF and serum biomarkers following single or (2-4x) PCI; results to date indicate elevations in serum GFAP, Tau and UCH-L1 with varying degrees of correlation to Righting Reflex and NSSR Scores.***
5. ***Added studies examining the expression of Cathepsin B following rPCI.***
6. ***Completed bioenergetic analyses of brain tissue and CSF following (2x) or (3x) PCI induced at different inter injury intervals.***
7. ***Completed sample collection to investigate microRNA biomarker profiles from serum.***
8. ***Completed sensorimotor analysis including performance on the CatWalk and Rotarod tasks following (2x) PCI induced at different inter injury intervals.***
9. ***Completed acute and subacute assessment of cognitive (MWM) function following (4x) PCI at 1hr injury intervals.***
10. ***Initiated studies of negative affect following (4x) PCI using the air-puff negative vocalization test, elevated plus maze test, and forced swim test; preliminary results indicate measures of negative affect using the air-puff negative vocalization test.***
11. ***Completed EEG analysis following (4x) PCI at 1hr injury intervals.***

REPORTABLE OUTCOMES

(All reportable outcomes since project inception are shown; those from the 2014-2015 funding year are shown in bold font):

1. Leung LY, Larimore Z, Holmes L, Cartagena C, McLoughlin S, Bustos F, Schmid K, Shear DA, Tortella FC. The WRAIR Projectile Concussive Impact Model: Effects of Impact Direction and Projectile Property. Military Health System Research Symposium 2013. Fort Lauderdale, Florida. August 2013. (Oral presentation)
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11. Boutte A, Abbatiello B, Grant S, Hook G, Hook V, Tortella F, Shear S. (2015) Brain cathepsin B is elevated in both mild-closed and severe-penetrating traumatic brain injury models. *Journal of Neurotrauma* 32:A-1-A-152; page A49.
12. Johnson D, Boutte A, Schmid K, Shear D, Dave J, Tortella F, Cartagena C (2015). Investigation of putative acute serum diagnostic biomarkers in a projectile concussive impact injury. *Journal of Neurotrauma* 32:A-1-A-152; page A36.
13. Deng-Bryant Y, Leung LY, Yang Y, Tortella F, Shear D (2015). Discovering prognosticators for repeated concussion: A global metabolomics study. *Journal of Neurotrauma* 32:A-1-A-152; page A36.
14. Lu XM, Cao Y, Liao Z, Tortella F, Shear D (2015). Time-course profile of EEG abnormality detected by qEEG power spectral analysis following a single concussive brain injury in rats. *Journal of Neurotrauma* 32:A-1-A-152; page A86.
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CONCLUSIONS

Phase I (SOW 1) studies designed to evaluate the time course effects of a single concussion on clinically relevant outcome measures have been completed. Phase II (SOW2) studies designed to evaluate the effects of repeated concussions that occur prior to and after the resolution of the healing profile for a single concussion were continued in Y3. Some aspects Phase II studies are complete while others are ongoing. Already, many valuable insights into the effects of repeated concussive injuries have been obtained from these studies. We found that in the majority, but not all, of parameters evaluated repeated concussion induced a greater degree of deficit and greater number of deficits compared to a single injury (e.g. altered levels of conscious, gait abnormalities, working memory deficits, metabolomics dysregulation, microglial phenotype alterations, and tissue and biofluid markers such as GFAP and tau). In addition, we found that the healing profile was often specific to each parameter with some parameters resolving within a day (e.g. EEG functional changes), while in others there was a delayed in the appearance of pathology for several days (e.g. tissue tau GFAP, SBDP pathology). Some parameters showed significant deficits sub-acutely or chronically post injury (e.g. gait abnormalities, working memory deficits). Interestingly, a few parameters showed a biphasic pattern where deficits resolved initially but reappeared later (e.g. gait abnormalities and metabolomics dysregulation). Evaluation of inter-injury intervals indicated that while a 24h inter-injury interval lead to a reduction in the number of positive CSF biomarker findings, changes in some tissue pathology parameters were as severe or showed greater severity (e.g. metabolomics dysregulation, cortical GFAP/ SBDP pathology). In contrast, with a 7 day inter-injury interval there was substantially reduced pathology, although deficits and pathology remain (gait abnormalities and metabolomics dysregulation). Thus, the PCI model effectively recapitulates many of the characteristic features of mTBI/concussion. The establishment of clear pathology seen with both single and repetitive concussion in the PCI model provides the necessary groundwork for future studies of treatment paradigms designed ameliorate the deleterious effects of impact concussion.

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APPENDICES

- A. Quad Chart
- B. Y3 Annual Report Tables and Figures

A Military-Relevant Model of Closed Concussive Head Injury: Longitudinal Studies Characterizing and Validating Single and Repetitive mTBI



DMRDP

PI: Deborah A. Shear

Org: Walter Reed Army Institute of Research (WRAIR)

Problem and Military Relevance

- **Problem:** Objective diagnostic tools and knowledge about what occurs in the brain following closed-head concussive mTBI are extremely limited, in part due to a lack of clinically relevant animal models of closed-head concussive injury.
- **Military Relevance:** 28% of U.S. military personnel sustaining at least one concussive mTBI event while deployed (Warden, 2006). Currently, over 150,000 military personnel have been diagnosed with a mild TBI. *Moreover, combat troops are often exposed to more than one concussion or mTBI in a short timeframe, the cumulative effects of which can produce long-lasting effects including physical, mental, emotional and cognitive impairments and may place our returning soldiers at increased risk for PTSD and/or neurodegenerative disorders.*

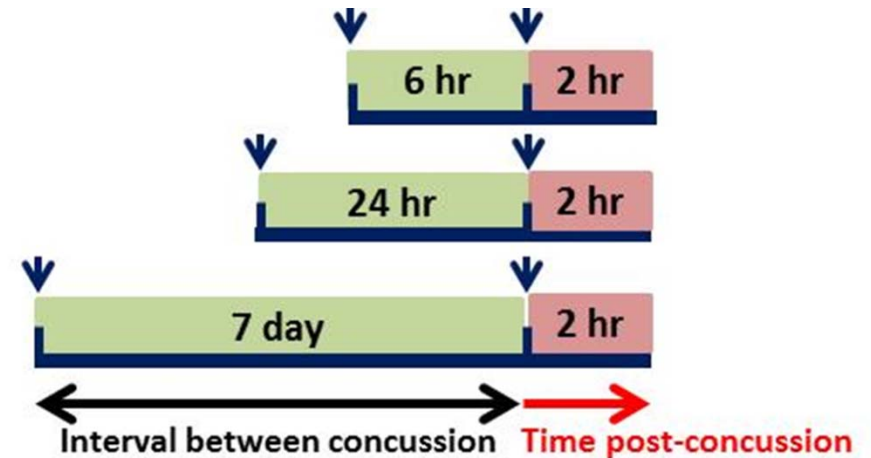


Diagram of Experimental Design for Evaluating Repeated Concussion occurring at Varied Intervals.

Proposed Solution

A fully characterized, validated animal model of close-head concussive mild TBI that can be used as a research tool to study potential treatment strategies and provide more objective diagnostic tools to improve guidelines for managing cerebral concussion.

- **Objective 1:** Fully characterize the effects of a “**SINGLE**” PCI across neuropathological, molecular and neurobehavioral outcome metrics to develop a “healing profile” demonstrating the time it takes the brain to return to “normal”.
- **Objective 2:** Evaluate the cumulative effects of “**REPEATED**” PCI injuries occurring *either before or after* resolution of the “healing profile”.

Timeline and Total Cost (direct and indirect)

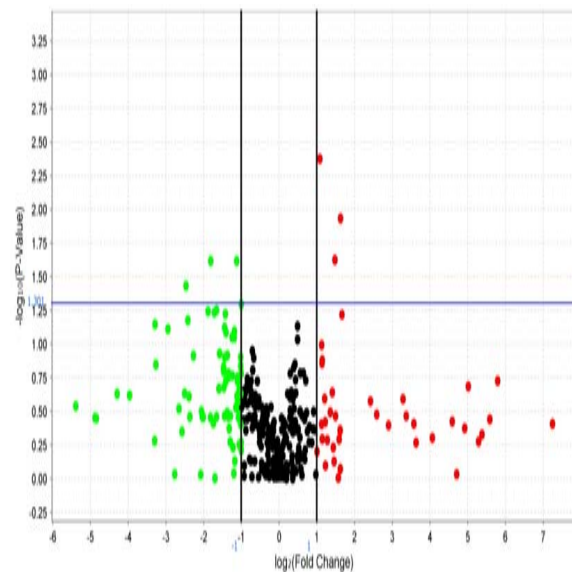
Activities	FY13	FY14	FY15	FY16
Phase 1: Comprehensive longitudinal characterization of a “SINGLE” PCI injury				
Phase 2: Evaluate of the cumulative effects of “REPEATED” PCI injuries				
Estimated Total Budget (\$K)	344K	365K	473K	505K

TABLE 1. Temporal Profile of Outcome Measures in PCI Model

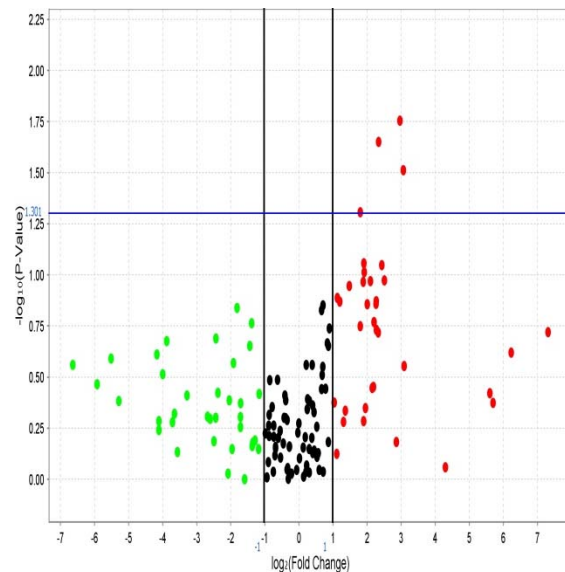
Outcome Measure	Single Concussion (sPCI)										Repeated Concussion (4xPCI, 1h interval)										
	1h	4h	6h	24h	48h	72h	7d	14d	1m	6m	1h	4h	6h	24h	48h	72h	7d	14d	21d	1m	6m
% change shown is vs. sham, *p < .05 vs. sham																					
Tissue Protein Levels (IHC)																					
APP (axonal)			+185%*	+22%		-31%	-65%						+1036%*	+429,96%*†		+33,403%*†		*			
GFAP (astrocyte)			+39%	+112%*		+52%	+35%						+74%*	-3%		+33%		ns			
Iba-1 (glial)			+65%*	+23%		+81%*	+27%						+88%*	+13%		-15%*		ns			
Claudin-5 (BBB)			-17%*	-9%		-19%*							-7%	-18%		-1%					
Aquaporin-4			-15%	+0.6%		-14%							tbd	-12%		+0.6%					
ZO-1			-5%	+3%		-2%							tbd	-19%		-41%*†					
Silver Staining (neurodegeneration)				+54%		+2%	-7%							+20.97%		+143.8%*†		*			
IBA1-M1 Phenotype			↓										↑ +62%			↑					
IBA1-M2 Phenotype			↑										↓ -14%			↓					
M1/M2 Ratio			↓										↑ +89%†			↑					
% change shown is vs. sham, *p < .05 vs. sham, *p < .05 vs. sham, †p < .05 vs sPCI																					
Tissue Protein Levels (Western)																					
GFAP/ GFAPbdp (Cx, HC)		ns		ns		ns	ns					ns		ns		+138%*† [CX]	ns				
UCH-L1		ns		ns		ns										+41% [CX]*†	ns				
SBPD (Cx, HC)		ns		ns		ns	ns							ns		ns	ns				
APP-FL(Cx, HC)		ns		ns		ns	ns							ns		ns	ns				
APP-betaCTFs (Cx, HC)		+39% [CX] †		ns		ns	ns							ns		ns	ns				
Tau (Cx, HC)		ns		ns		ns	ns							ns		+33% [HC]*†	ns				
p-Tau S202 (Cx, HC)		ns		ns		ns	ns							-17% [CX]*†		+36% [HC]*†	ns				
GFAP/ GFAPbdp (frontal CX)																					
% change shown is vs. sham, *p < .05 vs. sham																					
Metabolomic Profile																					
	30min	2h	6h	24h		72h	7d				2h										
% of total metabolite changed	11.1%*	12.4%*	14.9%*	2.2%*		3.7%*	6.8%*				17.1%*										
% change shown is vs. sham, *p < .05 vs. sham																					
Inflammatory Cytokines																					
TIMP	ns			400%*		ns	ns				400%*†			900%*†		300%*†	ns				
CINC	ns			ns		ns	ns				6000%*†			ns		ns	ns				
OTHER	ns			ns		ns	ns				350%*†			250%*†		ns	ns				
% change shown is vs. sham, *p < .05 vs. sham																					
CSF Biomarkers																					
GFAP (see footnote)	100%*	ns		20%*							0%*			ns							
UCH-L1(see footnote)	130%*	ns		ns							50%*			ns							
Tau	ns	ns		46%*							573%*†			2,076%*†							
p-Tau T 231	ns			ns							ns			ns							
% change shown is vs. sham, *p < .05 vs. sham																					
Serum Biomarkers																					
GFAP (see footnote)	ns			ns							100%*			ns							
UCH-L1(see footnote)	ns			ns							ns										
Tau	ns			100%*							ns										
p-Tau T 231	ns										ns										
miRNA (# of RNAs changed)		↑ 2 ↓ 2 *		↑ 4 *		↑ 4 *															
% change shown is vs. sham, *p < .05 vs. sham																					
Functional Outcomes																					
NSSR	66%*	65%*		58%		0					13%*	13%*		4.4%		9%					
BBB (Andrea please spell this out)	-10%*										-16%*										
Rotarod	-25%			-3%							-73%*			-45%*							
Catwalk	1,800%*			1,000%*		300%*	ns	200%*			100%*†			109%*†		800%*†	3,200%*†		1,000%*†		
MWM							ns	ns	ns	ns				*	*	ns	ns	*	ns	-91%*†	
qEEG (slowing)			+35%*	+42%*		ns	ns	ns													
Ultrasonic vocalization														46%*							

Footnote: For CSF/serum GFAP and UCHL-1, the "100%" reflects a comparison between a positive signal and a zero or below limit of detection value.

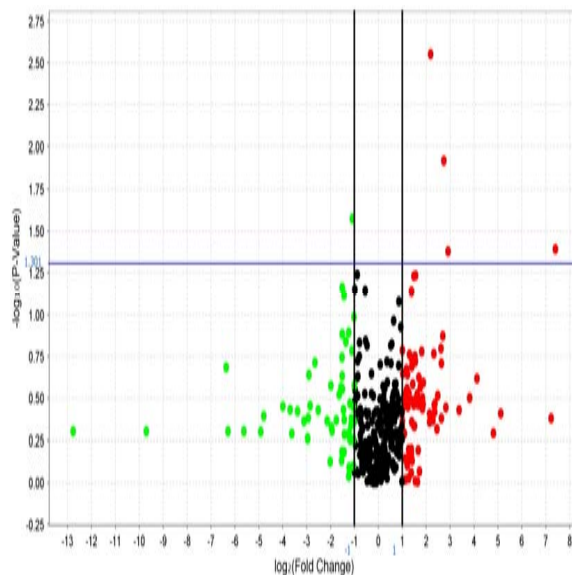
A. 4h Post PCI



C. 3d Post PCI



B. 1d Post PCI



D. 7d Post PCI

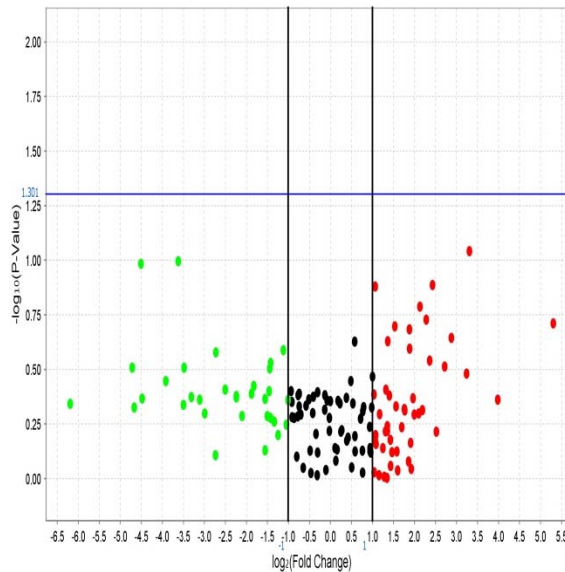


Figure 1. Volcano plot of Serum miRNAs (A) 4h post PCI. (B) 1d post PCI. (C) 3d post PCI. (D) 7d post PCI. Each dot represents one miRNA. Green dots are significantly decreased (top left quadrant), red dots (top right quadrant) are increased and black dots are unchanged. Horizontal blue line denotes level of significance ($p < 0.05$), where anything above is statistically significant compared to sham animals.

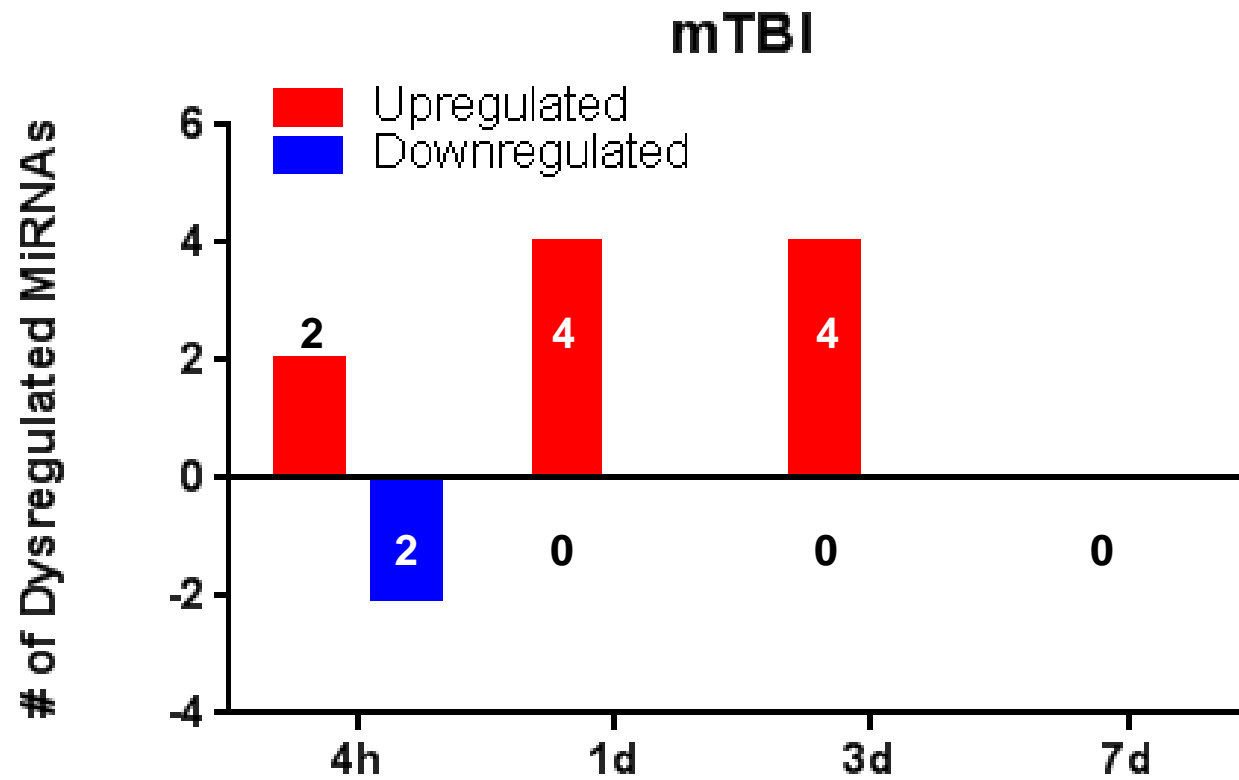


Figure 2. Dysregulated miRNAs in serum following single PCI injury.

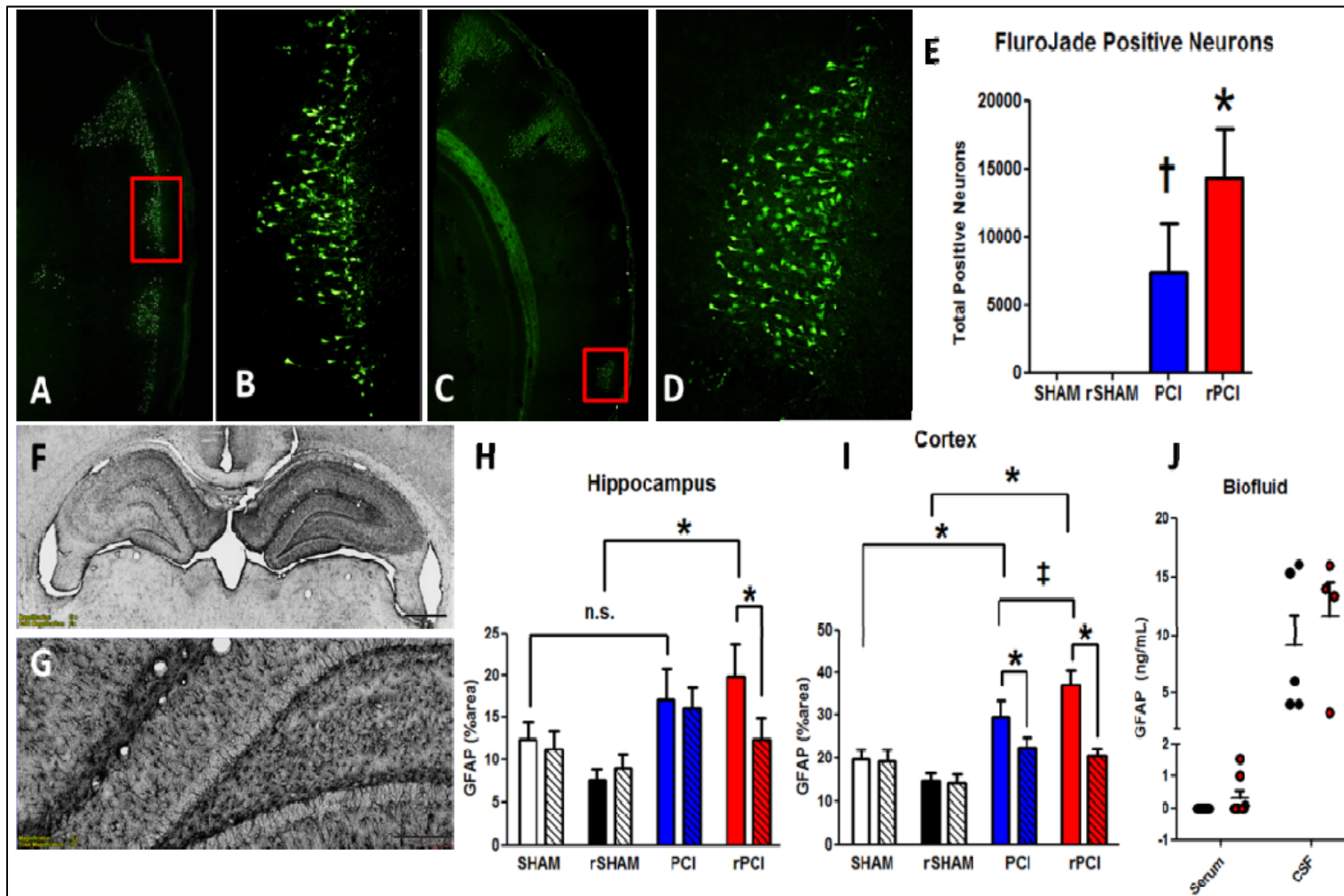


Figure 3. Repeated PCI increases Fluoro JadeB and GFAP levels in the brain 24h post-injury. Animals were sacrificed at 24h post-injury and brains were processed for Immunohistochemistry. Sections were stained for FluoroJadeB and GFAP (A, C) Brain section from rPCI animals showing discrete areas of Fluoro-Jade staining, 24h post-injury. (B, D) Expansion of insets. (E) Quantification of FluoroJadeB positive cells. (F) Brain section of PCI-injured animal, 24h-post injury showing asymmetric GFAP staining in hippocampus. (G) Expansion on hippocampus showing GFAP staining. Quantification of GFAP staining in Hippocampus (H) and Cortex (I); Open and lined bars represent ipsilateral and contralateral sides, respectively. (J) Quantification of GFAP levels in serum and CSF of rSHAM and rPCI animals. (* $p < 0.05$ rPCI vs rSHAM, ‡ $p < 0.05$ rPCI vs PCI, $n=6-8$ rat/group).

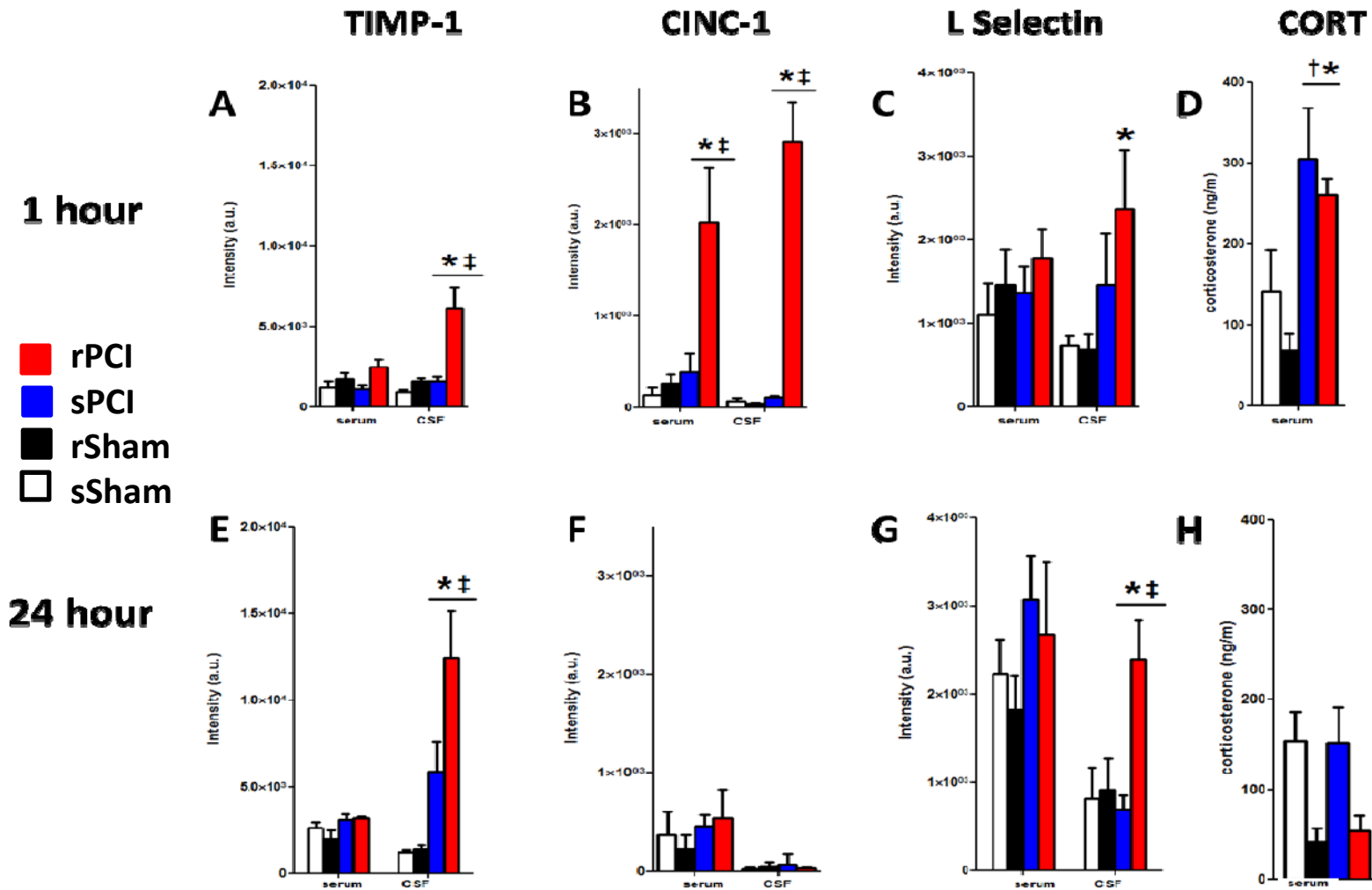


Figure 4. Repeated PCI increases neuroinflammation through 24h post-injury. Levels of inflammatory cytokines and chemokines were assessed in serum and CSF at 1 and 24h post-injury. RPCI resulted in significantly increased levels of TIMP-1 in CSF only at both 1 and 24h post-injury (A,E). In contrast, CINC-1 levels were elevated in both serum and CSF of rPCI at 1h post-PCI and subsequently returned to control levels. L-Selectin was significantly raised in rat CSF following repeated PCI at 1h post-injury which was sustained through 24h; whereas a trend was seen following a single PCI. No changes were evident in serum between groups. Corticosterone levels were significantly elevated following both single and repeated concussion (1h), but no difference between injured and respective sham was seen at later time points. (n=6-8rats/group/time point). (* $p < 0.05$ rPCI vs rSHAM, ‡ $p < 0.05$ rPCI vs PCI, n=8-10rat/group/time point).

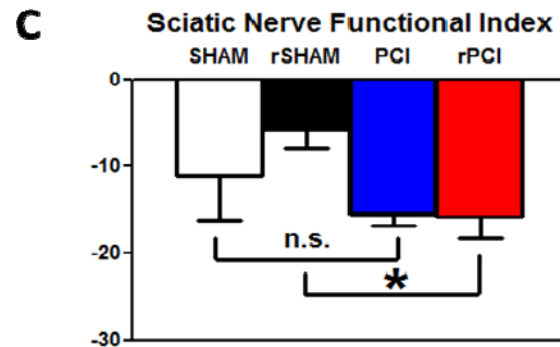
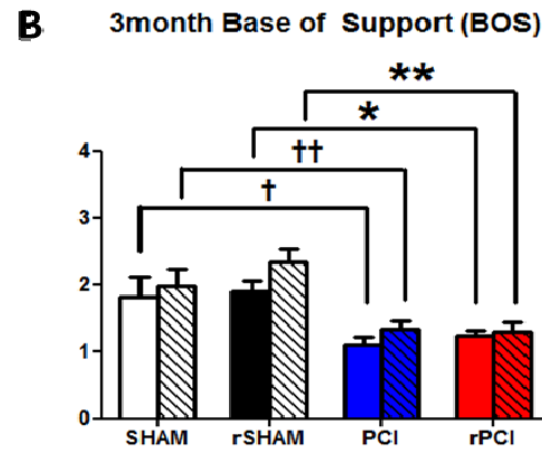
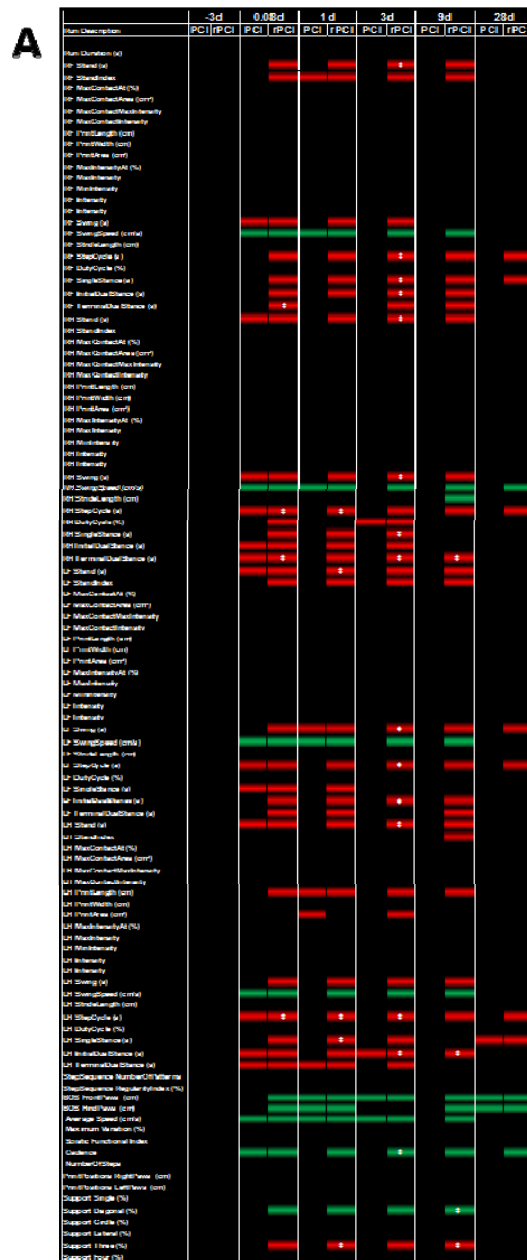


Figure 5. Single and repeated PCI results in acute and chronic gait abnormalities. Animals were tested for gait disturbances using the CatWalk automated gait analysis system. Both single and repeated PCI rodents displayed a significantly increased number of altered gait parameters which appeared to resolve over time. (A). Table highlighting significant differences in gait between sham, PCI and rSHAM and rPCI at various post-injury time points. Red bars denote parameters significantly increased whereas green bars denote significant decreases vs. sham. (A). The number of altered parameters was injury severity dependent. At three months post injury both single and repeated PCI rats showed significantly reduced BOS of both the front and hindlimbs (B). Repeated PCI rats also showed significantly altered Sciatic nerve Functional index (C). (n=6-8rats/group/time point). (* $p < 0.05$ rPCI vs rSHAM, † $p < 0.05$ PCI vs sham, n=8-10rat/group/time point).

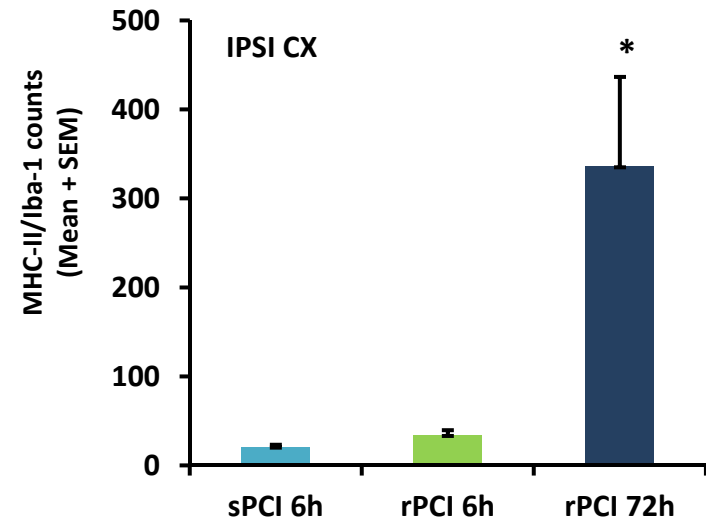
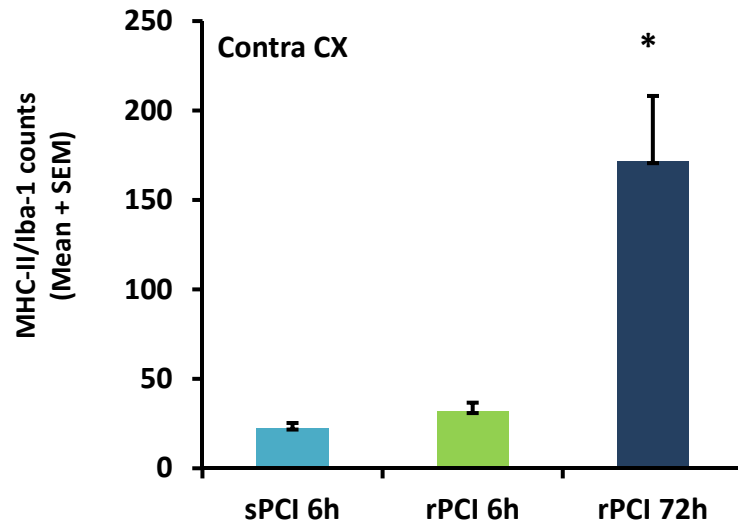


Figure 6. M-1 type microglial counts following single (sPCI) and repeat PCI (rPCI). * $p < 0.05$ compared to sPCI (TTEST)

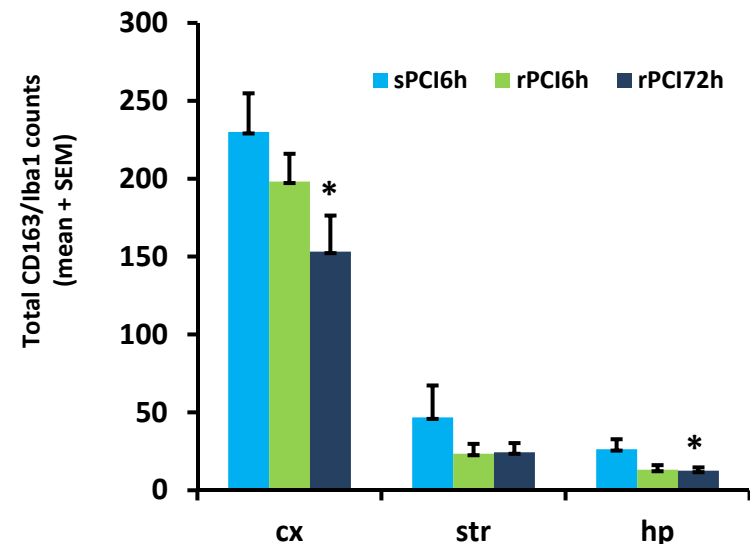
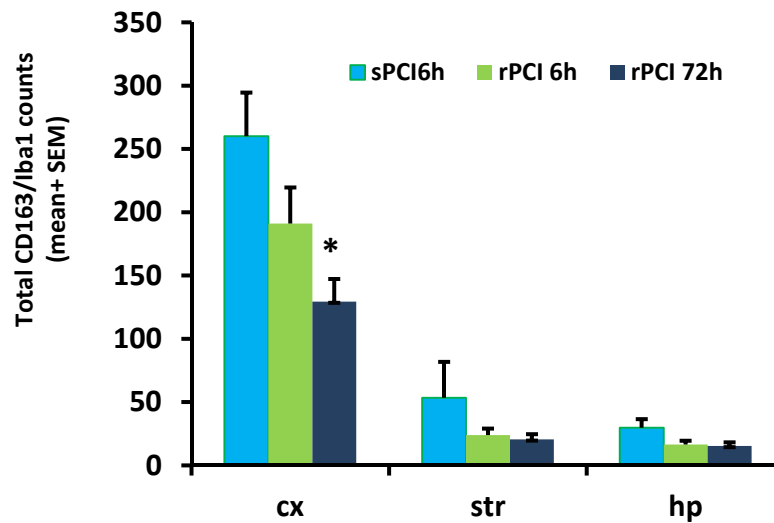


Figure 7. M-2 type microglial counts following single (sPCI) and repeat PCI (rPCI). Cx: cortex; str: striatum; hp:hippocampus. * $p < 0.05$ compared to sPCI (TTEST).

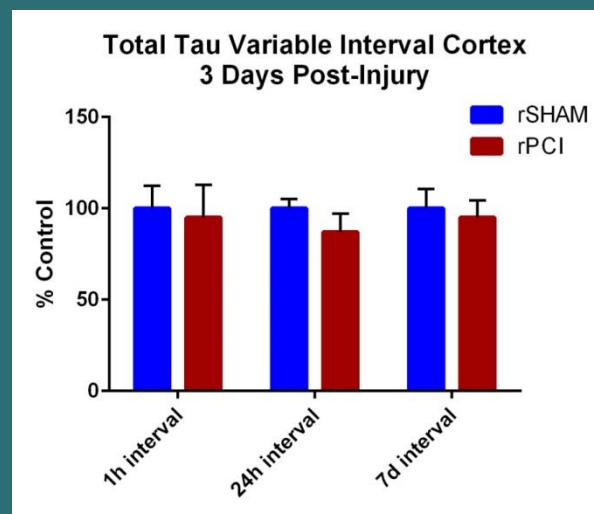
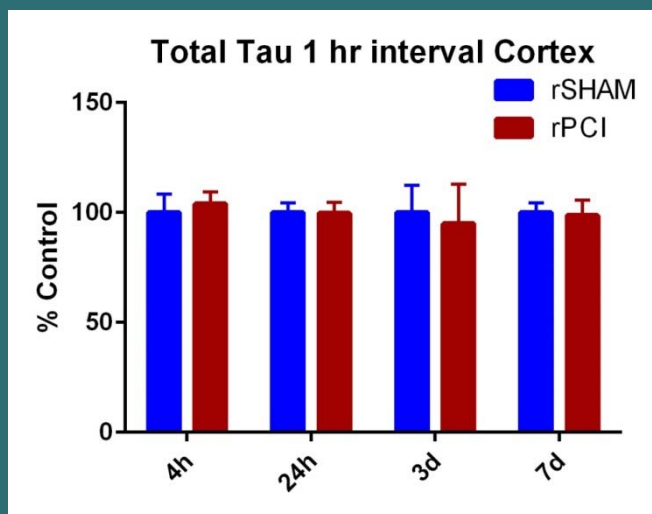
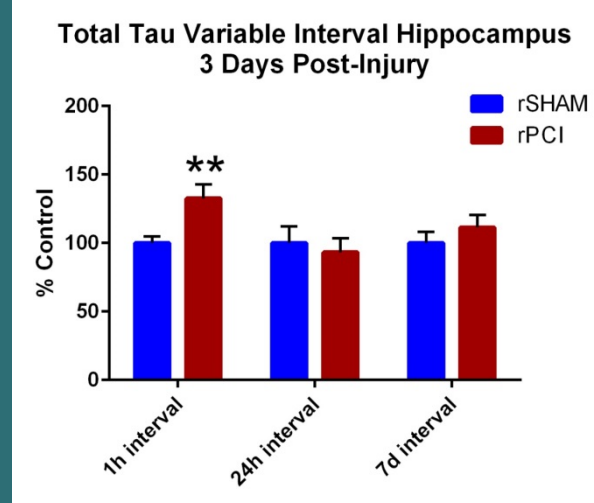
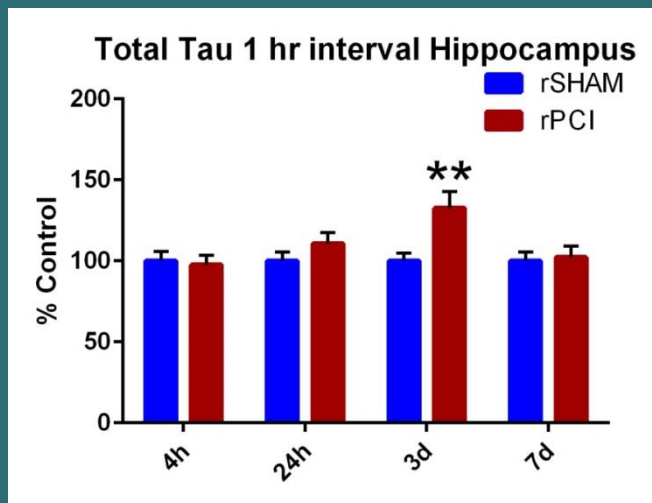


Figure 8. Increased Total Tau levels in HC 3 days post-injury.
 Student's t-test. ** $p < 0.01$ t-test of rPCI versus comparable sham. Error bars=SEM.

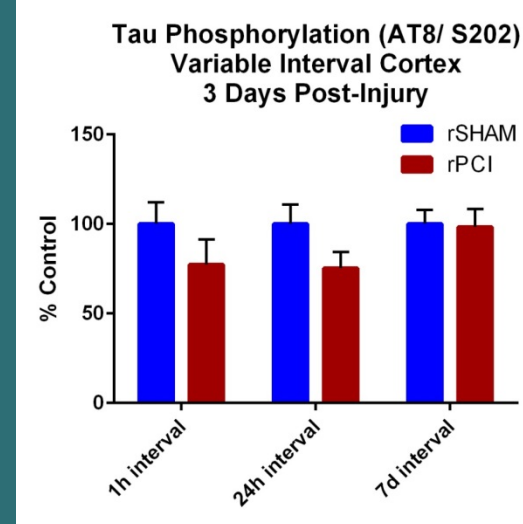
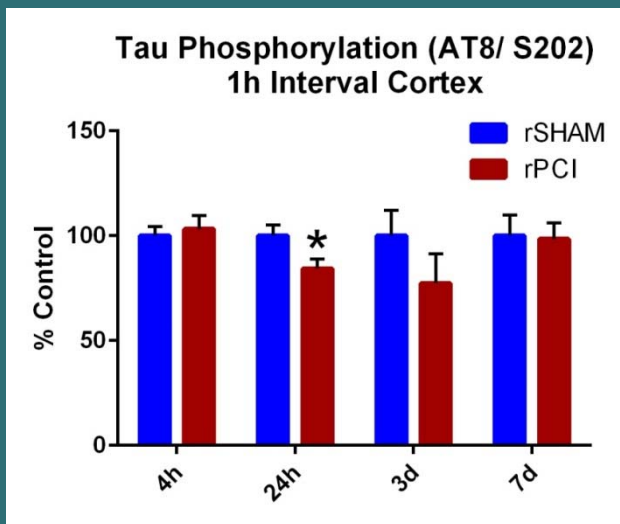
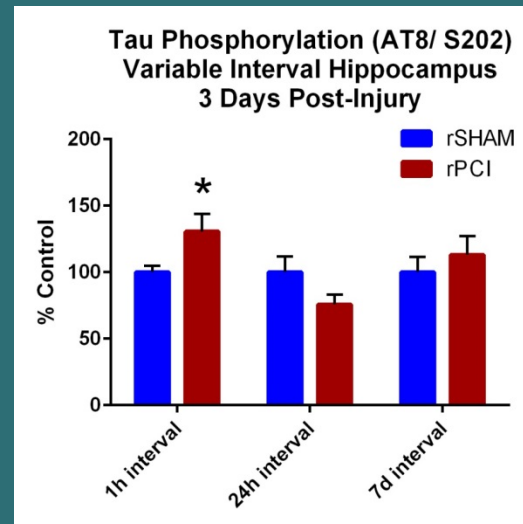
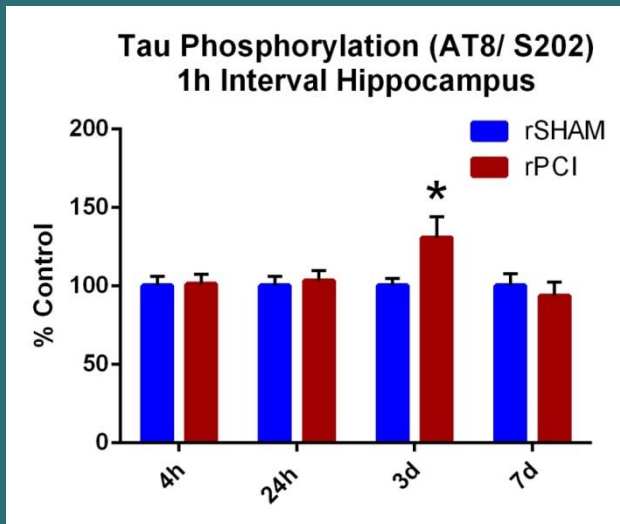


Figure 9. Increased pTau levels in HC 3 days post-injury. Student's t-test. ** $p < 0.01$ t-test of rPCI versus comparable sham. Error bars=SEM.

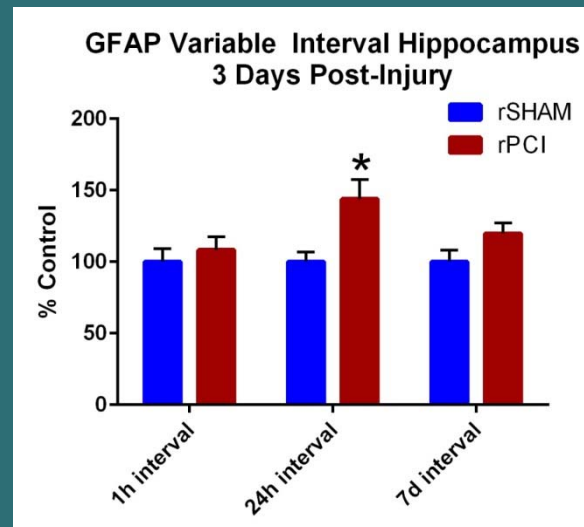
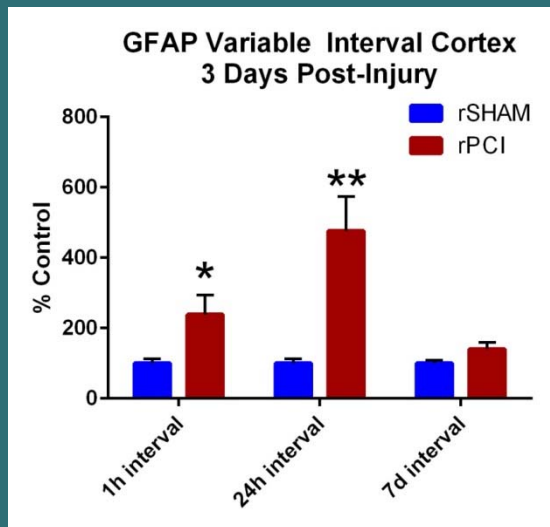
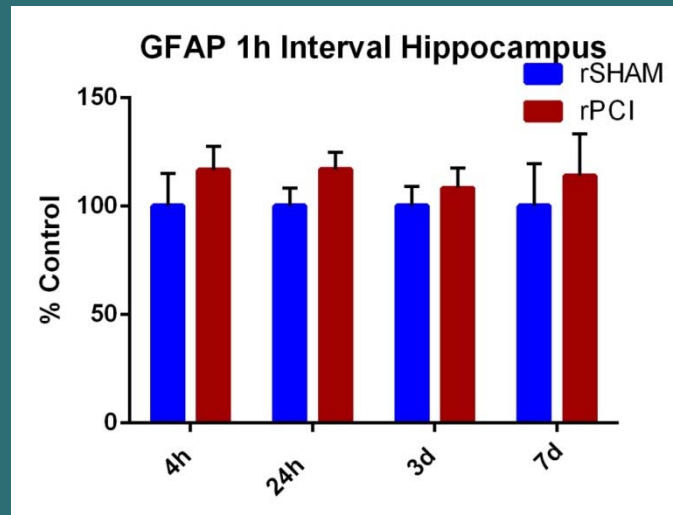
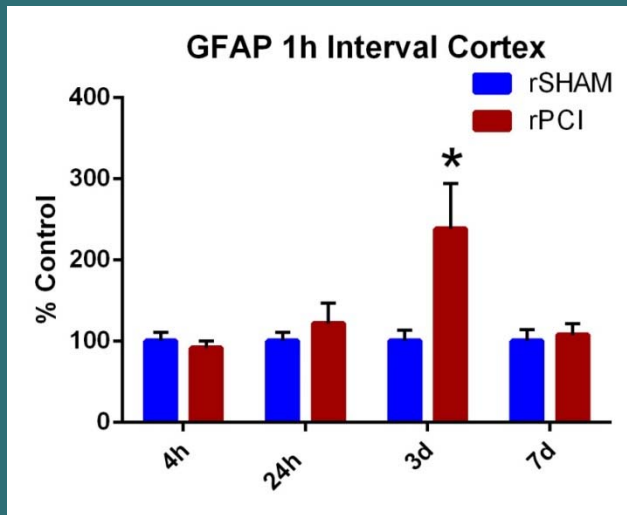


Figure10. Increased GFAP levels in CX and HC 3 days post-injury. Student's t-test. ** $p < 0.01$ t-test of rPCI versus comparable sham. Error bars=SEM.

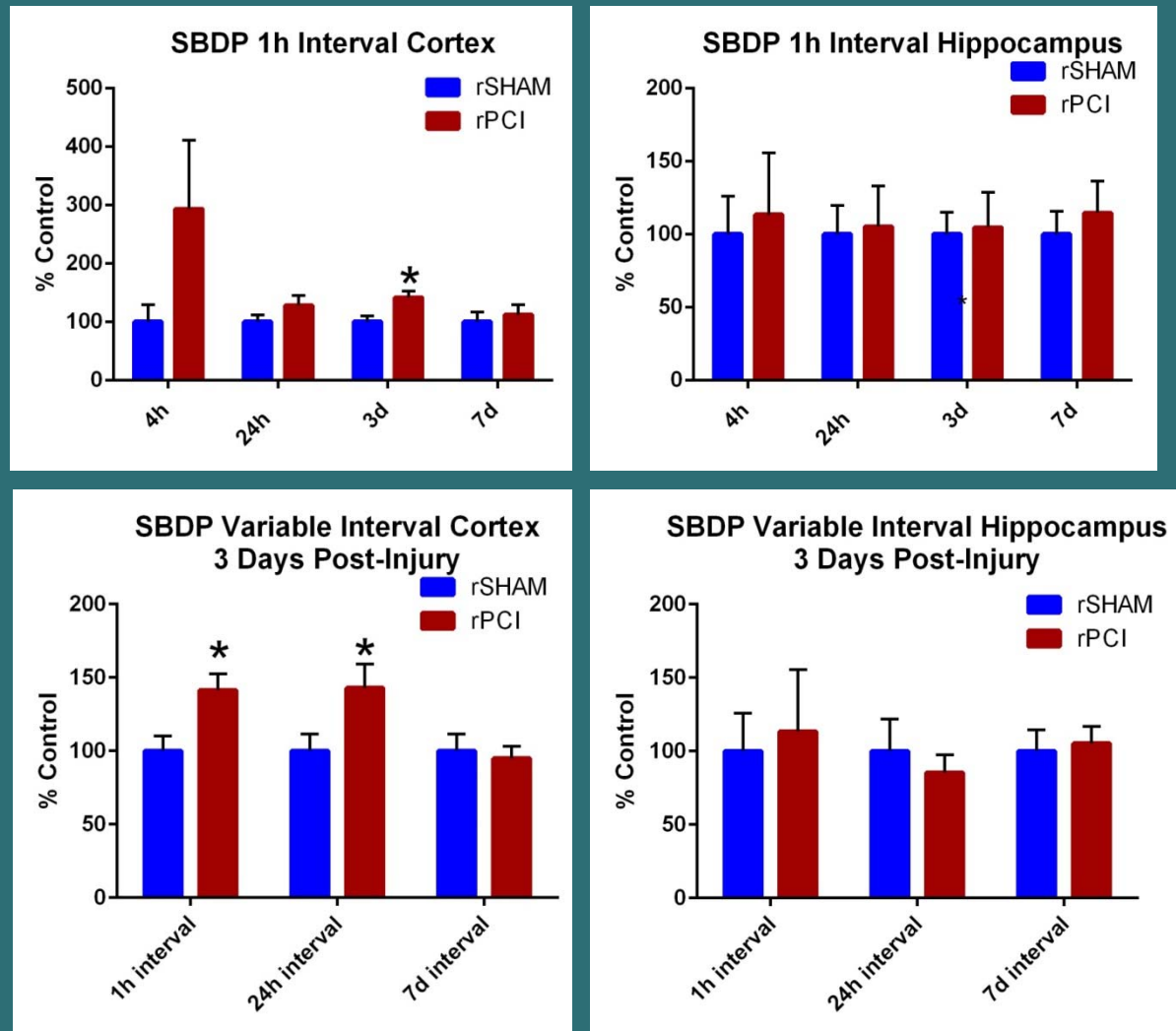


Figure 11. Increased SBDP levels in CX and HC 3 days post-injury. Student's t-test. ** $p < 0.01$ t-test of rPCI versus comparable sham. Error bars=SEM

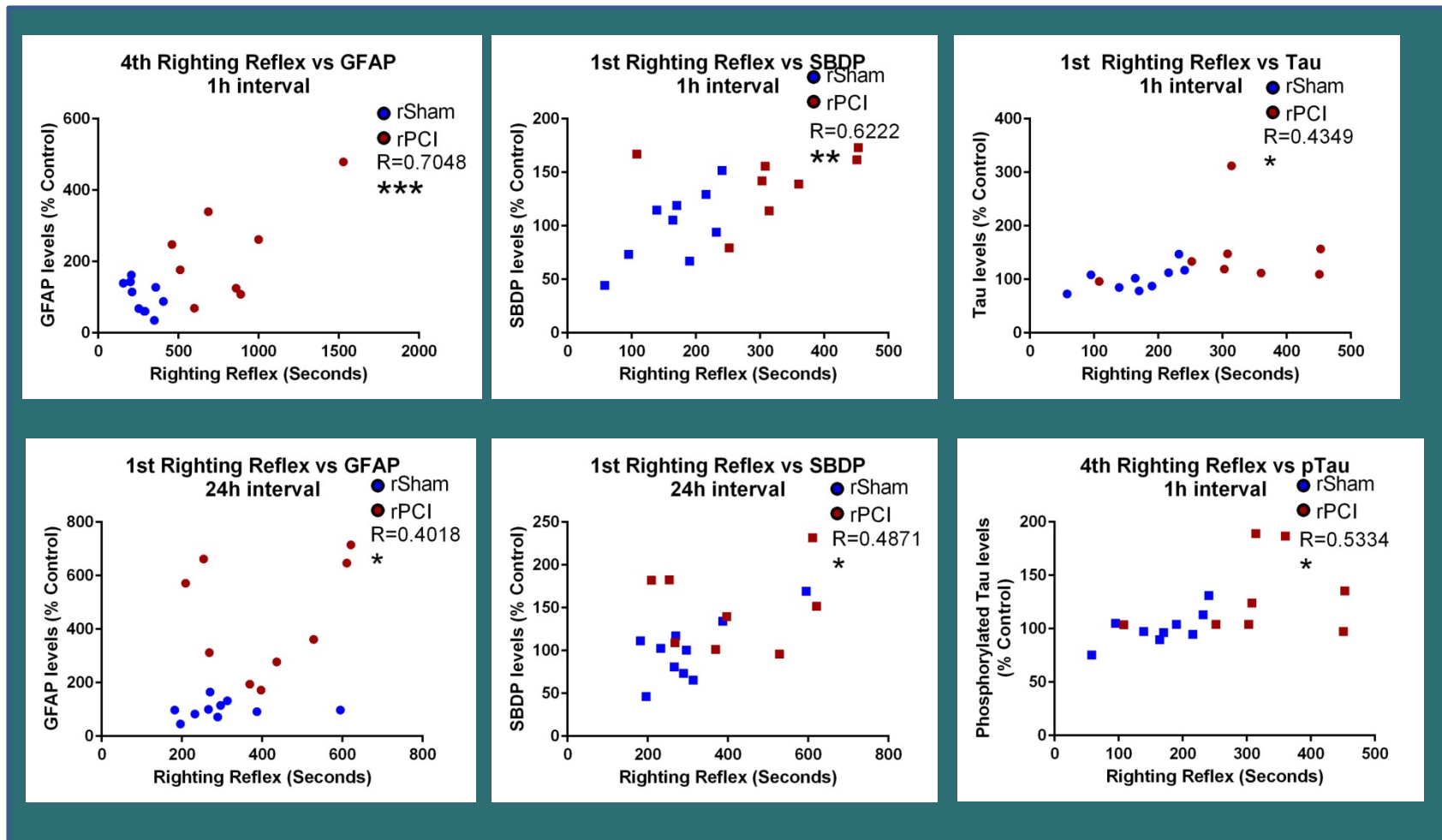


Figure 12. Correlations of Righting Reflex with Pathology Markers 3 days post-injury .
 Pearson's (one-tailed) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

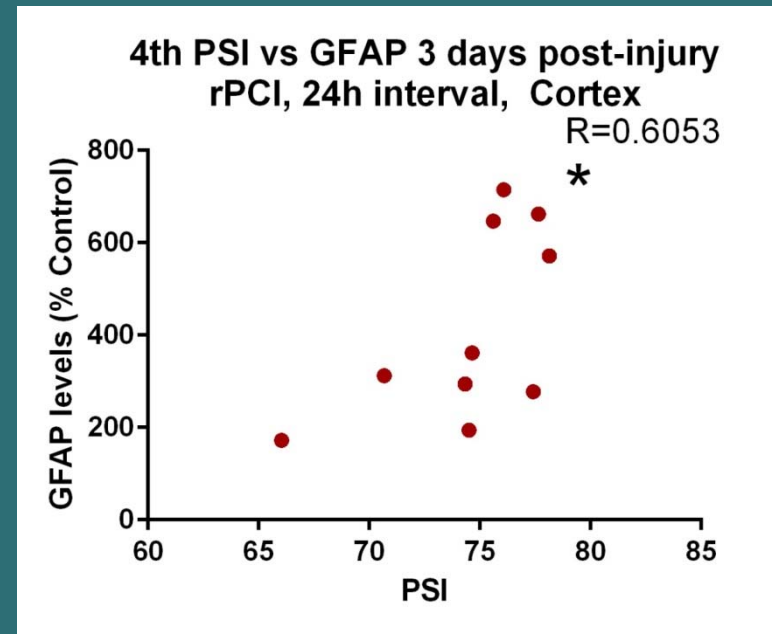
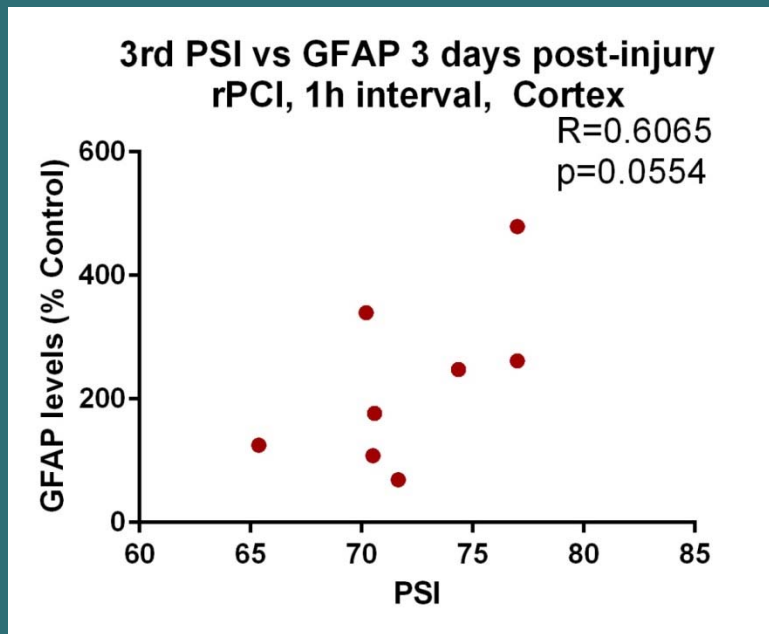


Figure 13. Correlations of Pressure inside helmet with GFAP 3 days post-injury. Pearson's (one-tailed) * $p<0.05$.

Results: Terminal Righting Reflex and Composite NSS-R Score per Injury Group

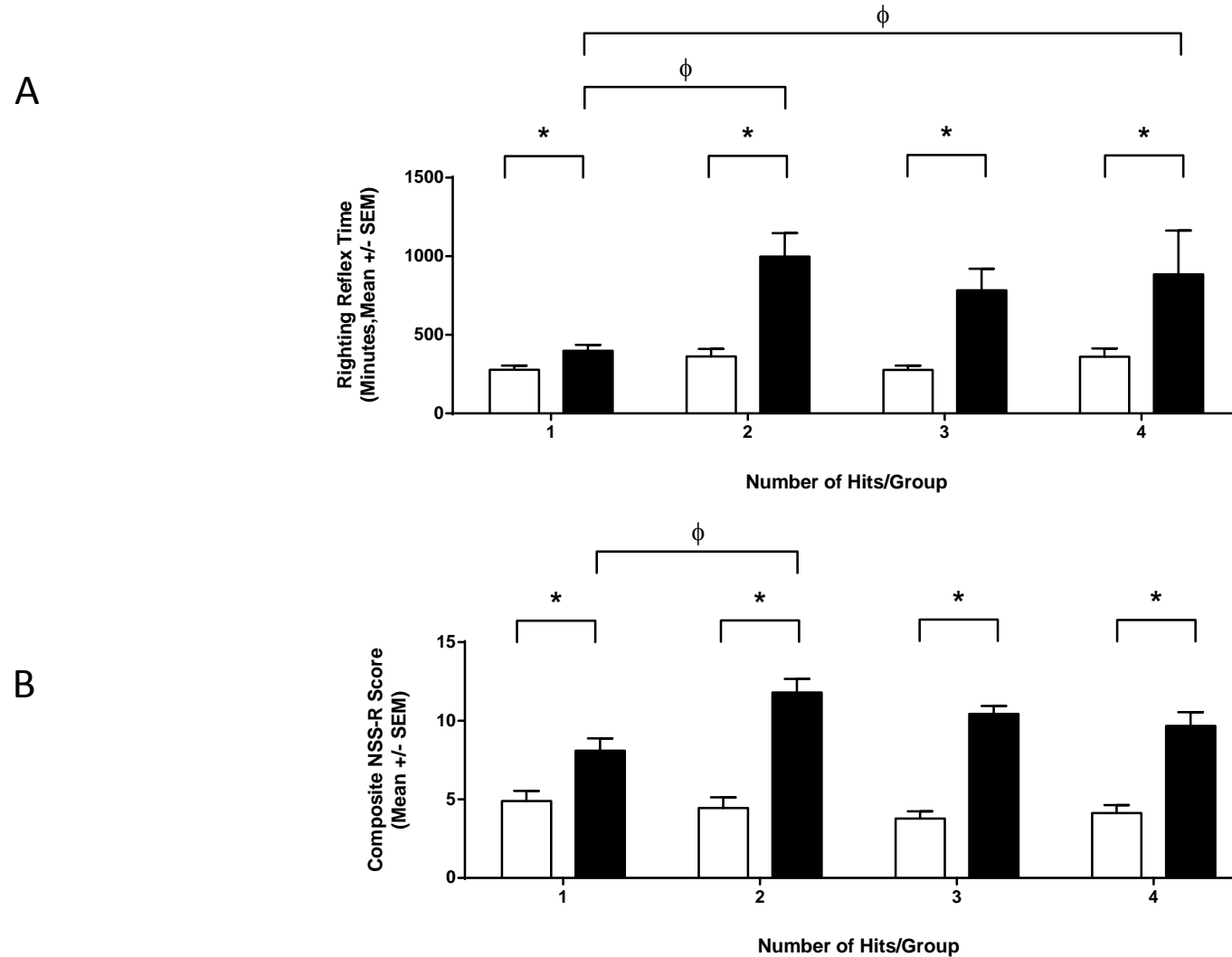


Figure 14A-B. Righting Reflex Time and Composite NSS-R Scores after Single or Repeated PCI. (A) The number of PCI hits (x-axis) and either (A) righting reflex time (RR) (y-axis, sec) or (B) the cumulative NSS-R score (y-axis) are displayed for each injury group. Sham (white bars) and PCI (black bars) are indicated for each single or repeated paradigm. Significance is noted by either asterisks (* $p \leq 0.05$ or ** $p \leq 0.001$, Student's t-test), or theta (Φ , 2-way ANOVA with the Tukey post-hoc test for effects between hits). *Note: there is little difference between repeated hit cohorts.*

Results: GFAP in CSF after Single and Repeated PCI

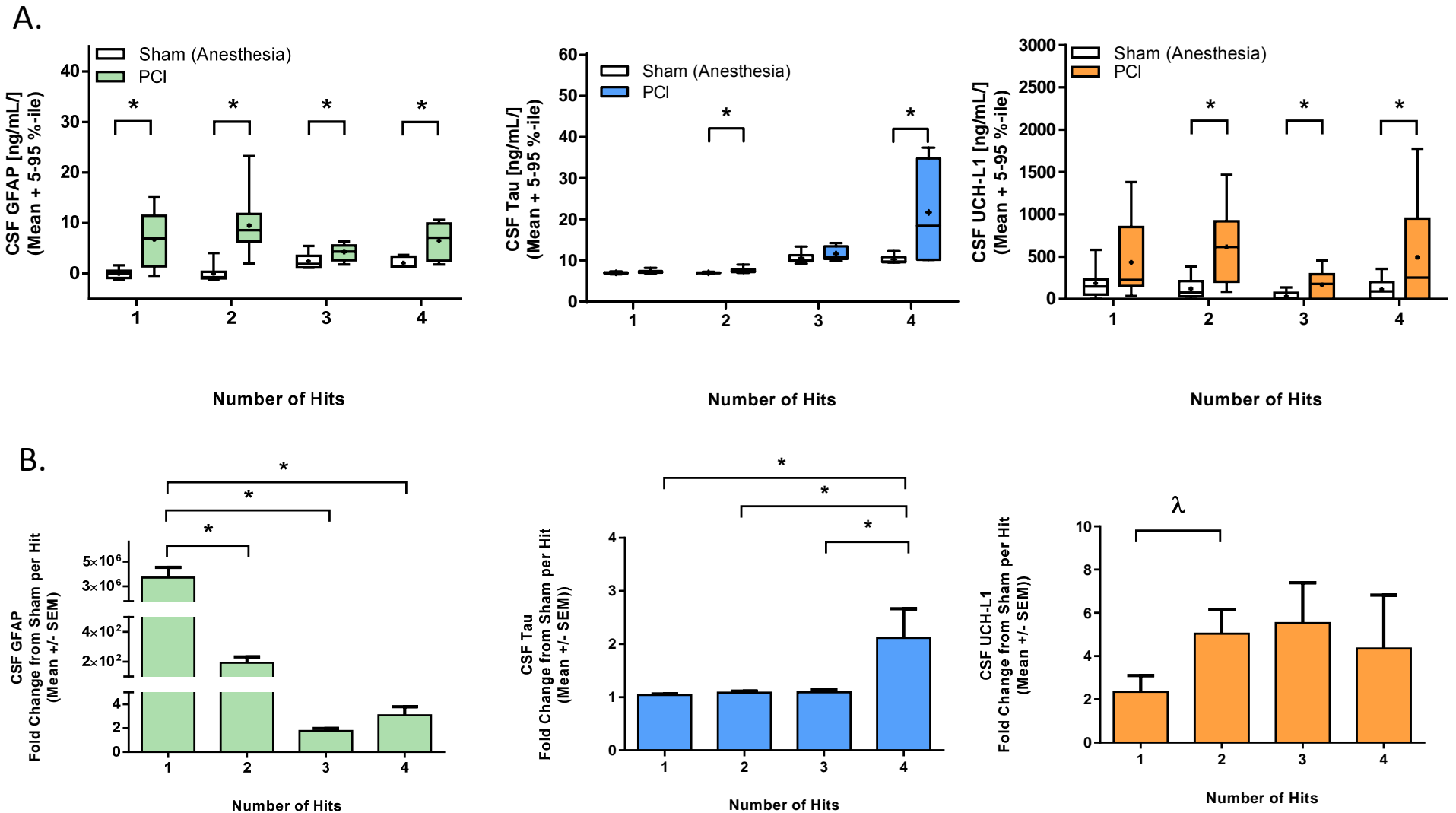


Figure 15. CSF Biomarker Profiles 1h after Single or Repeat PCI. (A) Quantitative profiles are displayed as the number of hits (x-axis) vs. the mean concentration [ng/mL] +/- 95% CI (y-axis) for each protein measured by MSD ELISA for GFAP (left), Tau (center), UCH-L1 (right). The number of hits (x-axis) and the mean fold change + SEM (y-axis) of PCI compared to its respective Sham control is shown. Significance is noted by asterisks (* $p \leq 0.05$, Student's t-test, PCI vs Sham control). (B) Fold-change transformed data from PCI groups compared to Sham is displayed for GFAP (left), Tau (center), UCH-L1 (right). Significance is noted by either an asterisk (*, $p \leq 0.05$ 1-way ANOVA with Fisher's Least Significance (LSD) test) or a lambda symbol (λ , $p \leq 0.05$ Student's t-test).

Results: Biomarker – RR Correlations

Shown as single v repeated, PCI Only

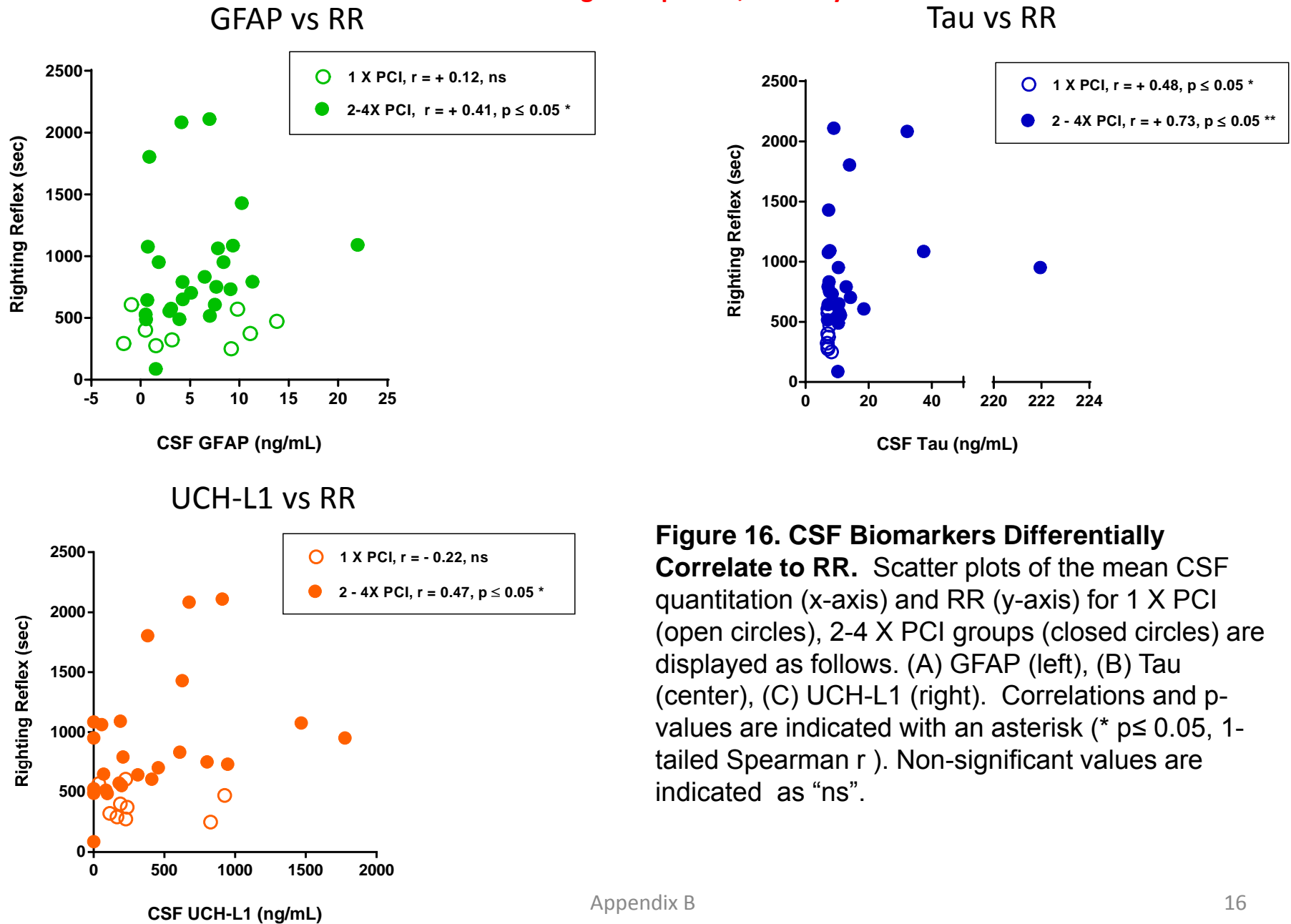
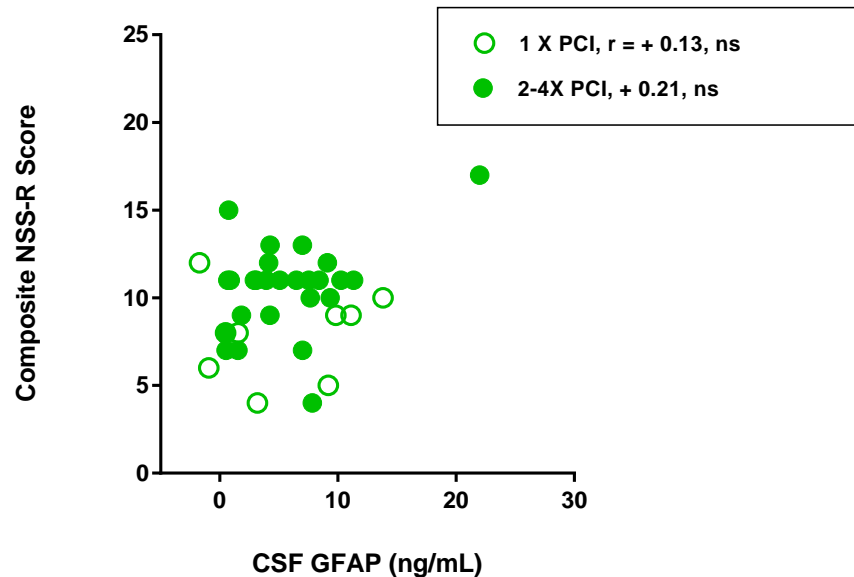


Figure 16. CSF Biomarkers Differentially Correlate to RR. Scatter plots of the mean CSF quantitation (x-axis) and RR (y-axis) for 1 X PCI (open circles), 2-4 X PCI groups (closed circles) are displayed as follows. (A) GFAP (left), (B) Tau (center), (C) UCH-L1 (right). Correlations and p-values are indicated with an asterisk (* $p \leq 0.05$, 1-tailed Spearman r). Non-significant values are indicated as “ns”.

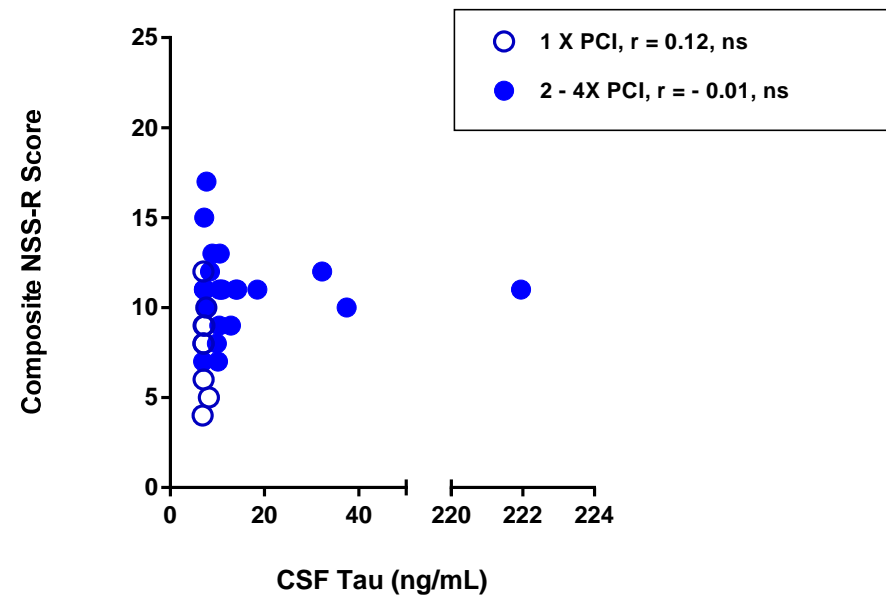
Results: Biomarker – Composite NSS-R Correlations

Shown as Single vs Repeated, PCI Only

GFAP vs cNSS-R



Tau vs cNSS-R



UCH-L1 vs cNSS-R

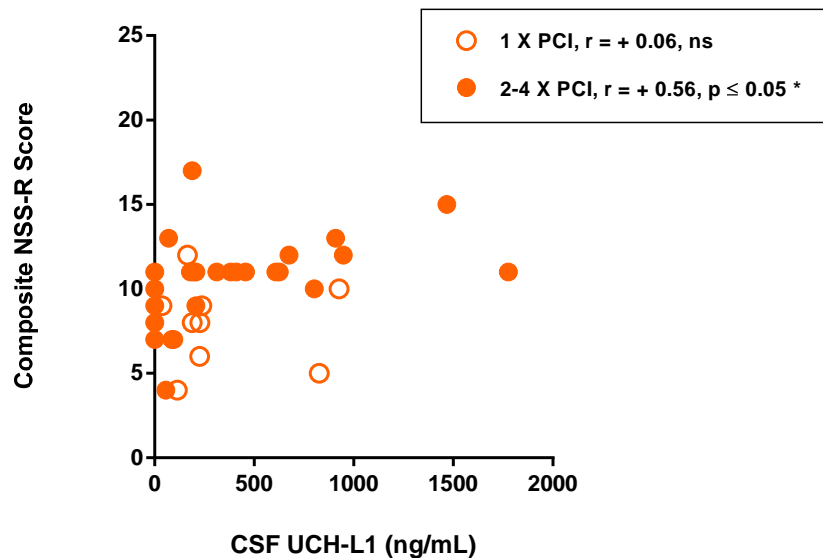


Figure 17. CSF Biomarkers Differentially Correlate to Composite NSS-R. Scatter plots of the mean CSF quantitation (x-axis) and NSS-R (y-axis) for 1 X PCI (open circles), 2-4 X PCI groups (closed circles) are displayed as follows. (A) GFAP (left), (B) Tau (center), (C) UCH-L1 (right). Correlations and p-values for significant groups are indicated with an asterisk (* $p \leq 0.05$, 1-tailed Spearman r). Non-significant values are indicated “ns”.

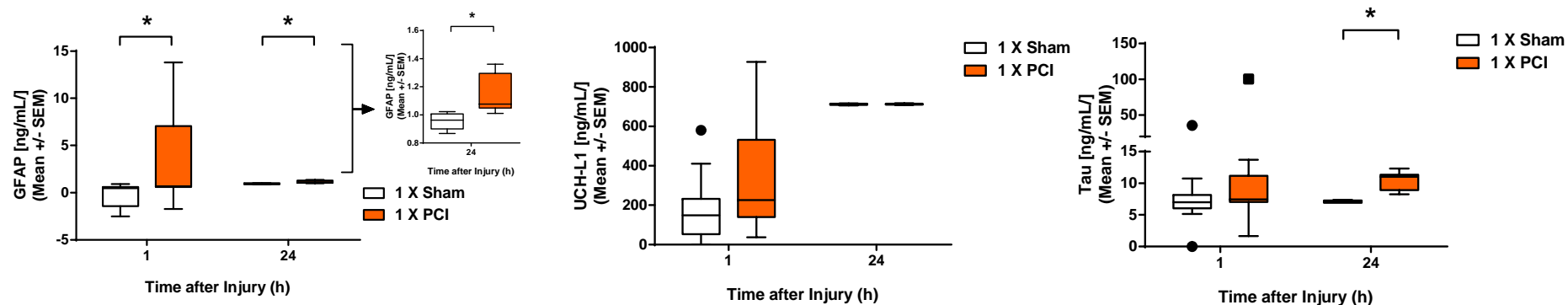


Figure 18. CSF Biomarkers Display Differential Temporal Patterns after Single, 1X, PCI with 1h Intervals. Box and whisker plots indicating the mean CSF quantitation (x-axis) over time (y-axis) for GFAP (left), UCH-L1 (center), and Tau (right) after 1 X PCI. 1XSham (white) and 1XPCI (orange) groups are indicated. Significant differences in PCI-Sham comparisons are indicated with an asterisk (* $p \leq 0.05$, 1-tailed Student's t-Test).

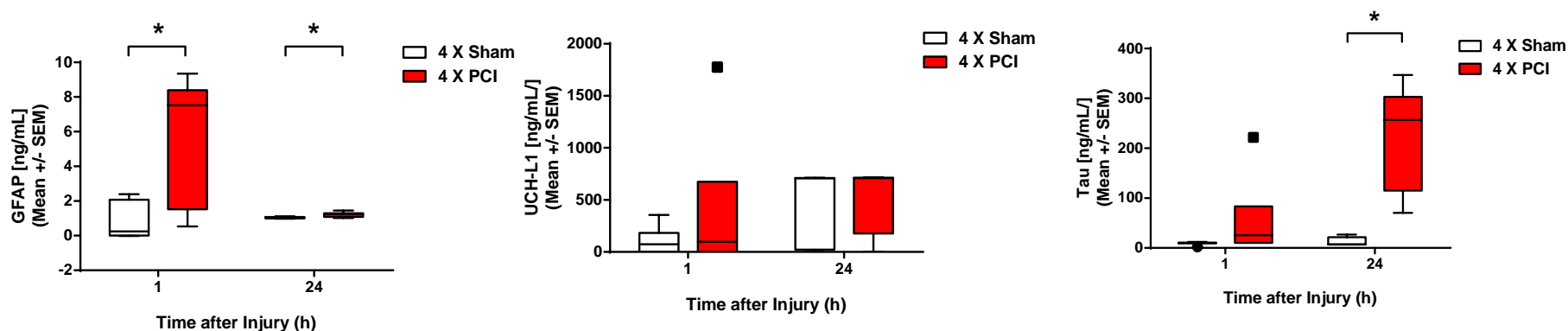


Figure 19. CSF Biomarkers Display Differential Temporal Patterns after Repeated, 4X, PCI with 1h Intervals. Box and whisker plots indicating the mean CSF quantitation (x-axis) over time (y-axis) for GFAP (left), UCH-L1 (center), and Tau (right) after 4 X PCI. 4XSham (white) and 4XPCI (red) groups are indicated. Significant differences in PCI-Sham comparisons are indicated with an asterisk (* $p \leq 0.05$, 1-tailed Student's t-Test).

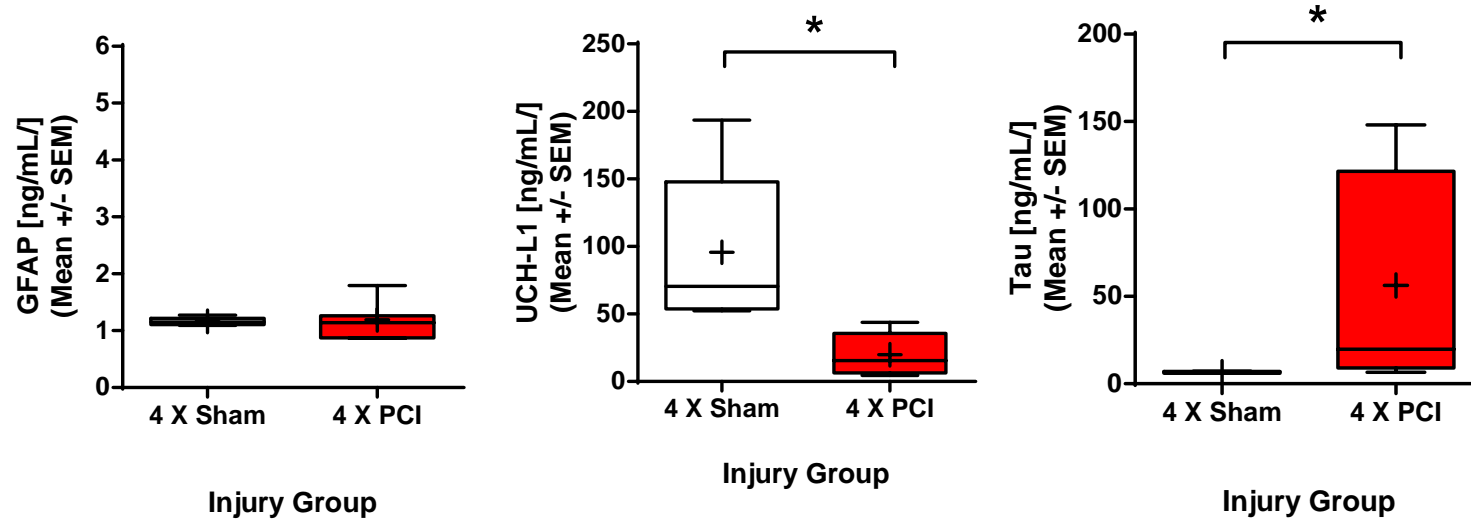


Figure 20. CSF Biomarkers Display Differential Patterns after 4XPCI with 24h Intervals. Box and whisker plots indicating the mean CSF quantitation (x-axis) over time (y-axis) for GFAP (left), UCH-L1 (center), and Tau (right) after 4 X PCI. 4XSham (white) and 4XPCI (red) groups are indicated. Significant differences in PCI-Sham comparisons are indicated with an asterisk (* $p \leq 0.05$, 1-tailed Student's t-Test).

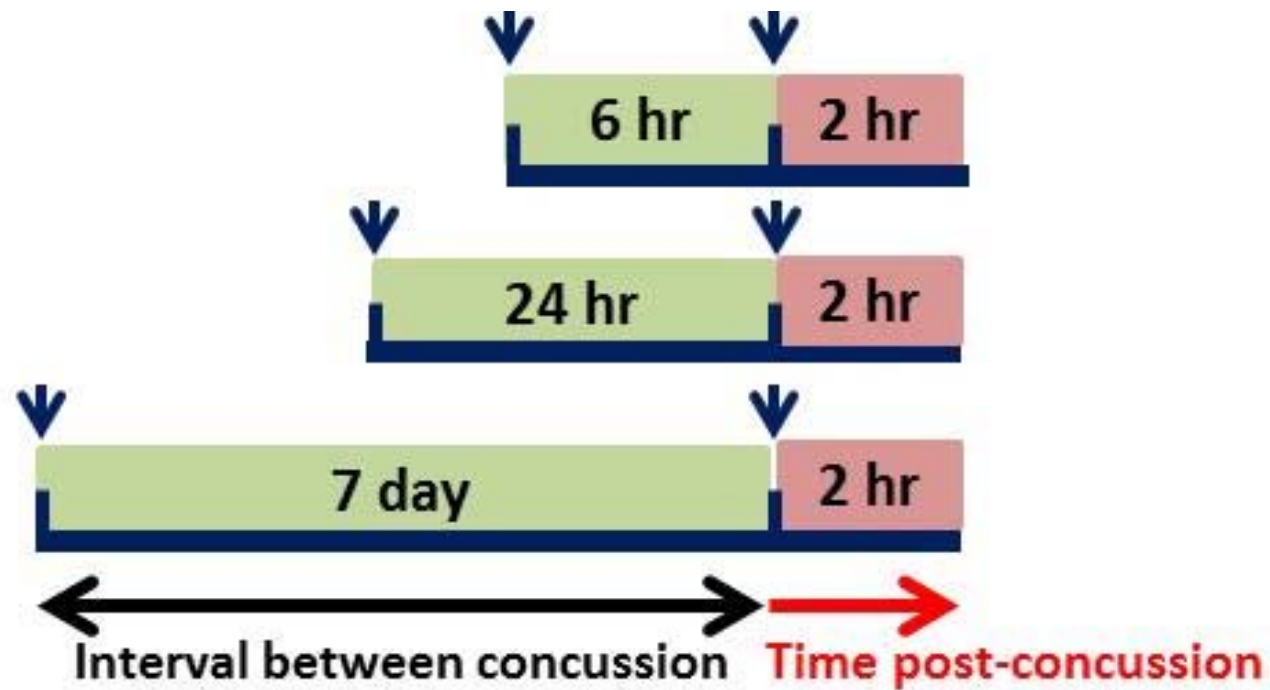


Figure 21. Diagram of experimental design.

Percent Metabolite Significantly Changed per Total Number of Metabolite Detected (vs. Sham control)

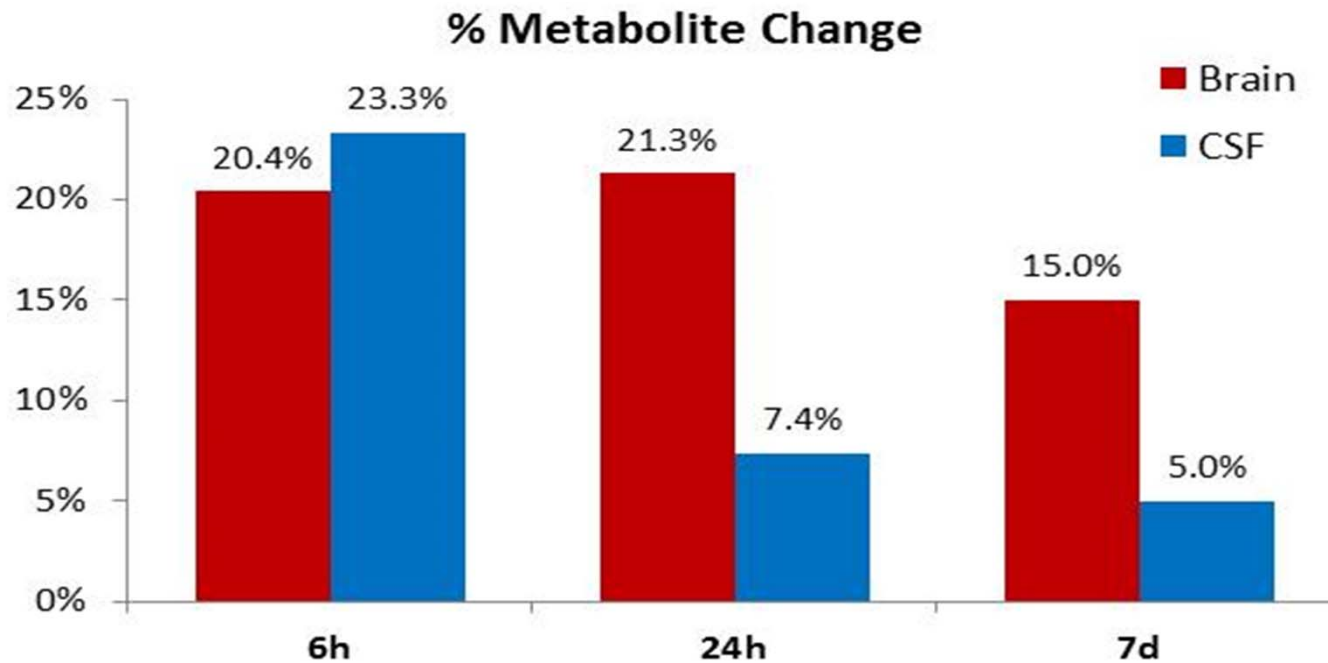


Figure 22. The percentage of biochemicals that were significantly altered following $2\times\text{PCI}_{6\text{h}}$, $2\times\text{PCI}_{24\text{h}}$, and $2\times\text{PCI}_{7\text{d}}$ were 20.4%, 21.3%, 15.0% in the brain ($p<.05$ vs. sham), and 23.3%, 7.4%, 5.0% in the CSF ($p<.05$ vs. sham).

Statistical Comparison - ANOVA Contrasts		Brain Tissue			
Significantly Altered Biochemicals		Total biochemicals $p \leq 0.05$	Biochemicals ($\uparrow \downarrow$)	Total biochemicals $0.05 \leq p \leq 0.10$	Biochemicals ($\uparrow \downarrow$)
<i>PCI</i> <i>Sham</i>	1XPCI	40	4 36	19	0 19
	2XPCI (6h interval)	94	62 32	40	20 20
	3XPCI (6h interval)	75	59 16	42	28 14

Figure 23. The results show significant alterations of biochemicals at 12.4%, 20.4% and 19.2% out of all biochemicals detected following 1xPCI, 2xPCI and 3xPCI ($p < 0.05$ vs. sham) respectively.

PARAMETER	PAW	PCI					2xPCI24					2xPCI7d				
		2hr	24hr	72hr	7d	30d	2hr	24hr	72hr	7d	30d	2hr	24hr	72hr	7d	30d
Speed																
Cadence																
# of Steps																
Sequence Regularity																
Base of Support, FRONT																
Base of Support, HIND																
Print Positions, RIGHT																
Print Positions, LEFT																
STAND	RF															
	LF															
	RH															
	LH															
STAND INDEX	RF															
	LF															
	RH															
	LH															
MAX CONTACT	RF															
	LF															
	RH															
	LH															
MAX INTENSITY	RF															
	LF															
	RH															
	LH															
SWING	RF															
	LF															
	RH															
	LH															
SWING SPEED	RF															
	LF															
	RH															
	LH															
STRIDE LENGTH	RF															
	LF															
	RH															
	LH															
STEP CYCLE	RF															
	LF															
	RH															
	LH															
DUTY CYCLE	RF															
	LF															
	RH															
	LH															
SINGLE STAND	RF															
	LF															
	RH															
	LH															
INITIAL DUAL STANCE	RF															
	LF															
	RH															
	LH															
TERMINAL DUAL STANCE	RF															
	LF															
	RH															
	LH															
PHASE DISPERSIONS	RF->LH															
	LF->RH															
	LH->RH															
	LF->RF															
	RF->LH															
COUPLINGS	RF->LH															
	LH->RH															
	LF->RF															
	LF->RH															
	RF->LH															
	RF->RH															
	LF->LH															
	RH->RF															
SUPPORT %	DIAGONAL															
	DIAGONAL															
	LATERAL															
	THREE															
	FOUR															

Figure 24. Changes in gait parameters following either single or repeated PCI (2xPCI24h, 2xPCI7d) with respect to corresponding control injury. Red indicates a significant increase, Green indicates a significant decrease (One-way Repeated Measures ANOVA; Holm-Sidak versus control $p < .05$)

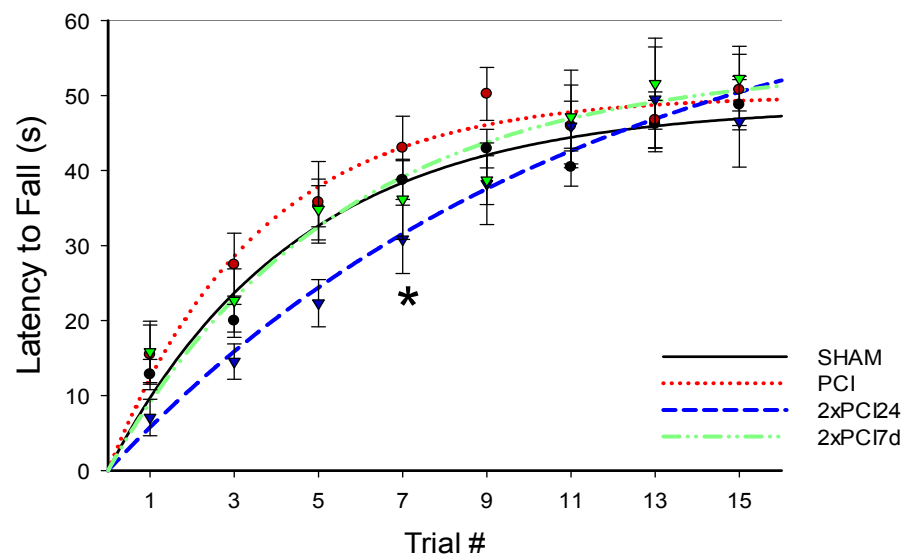


Figure 25. Rotarod performance at 2 months post-injury. Rats experiencing two PCI injuries spaced 24hrs apart displayed significant deficits in motor learning capacity compared to all other groups (non-linear regression, extra sum of squares F-test, $* < .01$).

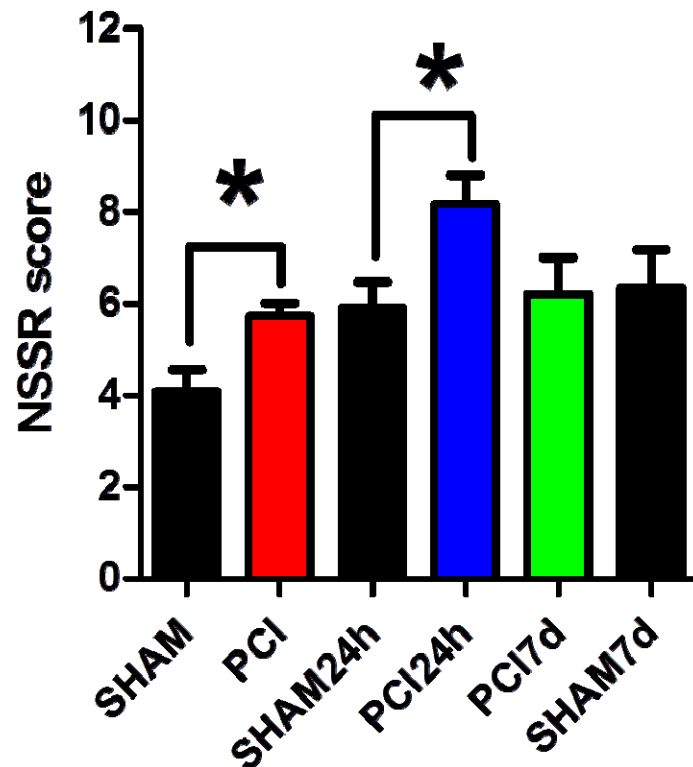


Figure 26. Mean composite NSSR scores at 3 months post-injury. Single PCI and 2xPCI24h displayed significant deficits compared to their respective SHAM animals (One-way ANOVA, $* p < .05$)

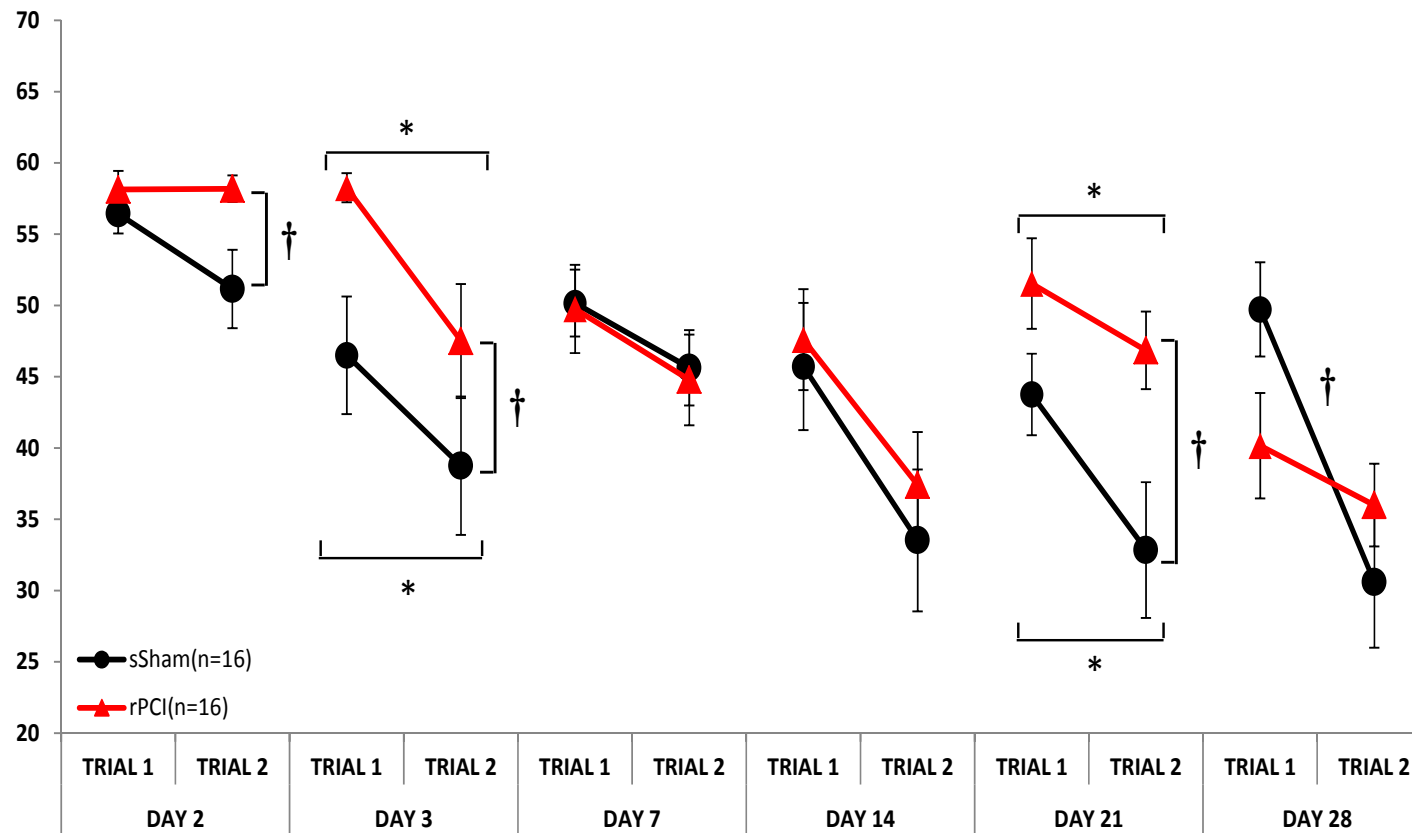


Figure 27. Working memory version of MWM was conducted at 2, 3, 7, 14, 21, and 28 days post-injury. Data was expressed as mean ± STE (n=16/group). P<0.05 was considered significant.

Air-Puff Induced Negative Vocalizations: 4 Hit PCI, 48 Hour Post-Injury

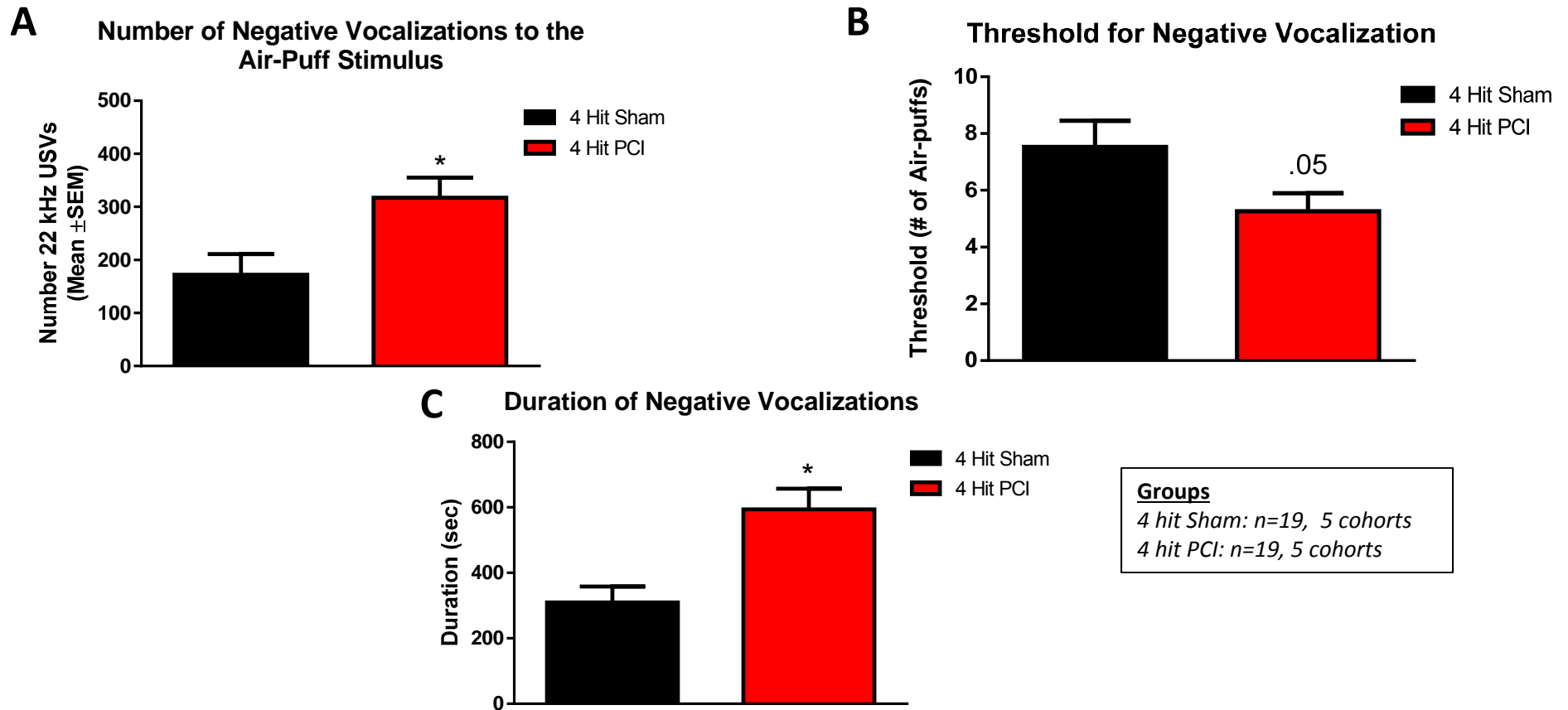


Figure 28. Four hit PCI leads to greater levels of negative affect when compared to shams. Four hit PCI led to the production of significantly more negative (22kHz) vocalizations in response to 15, 55psi air-puffs spaced 30secs apart (A). The number of air-puffs required for negative vocalization (threshold) shows a strong trend toward significance, with the PCI animals vocalizing to fewer air-puffs (B). The duration of negative vocalization was longer in the PCI animals compared to sham (C). Taken together these results indicate that the PCI animals have show a greater level of negative affect to the same mild stressor. * $p < .05$, Student's t-test

Elevated Plus Maze:

4 Hit PCI, 3 Day Post-Injury

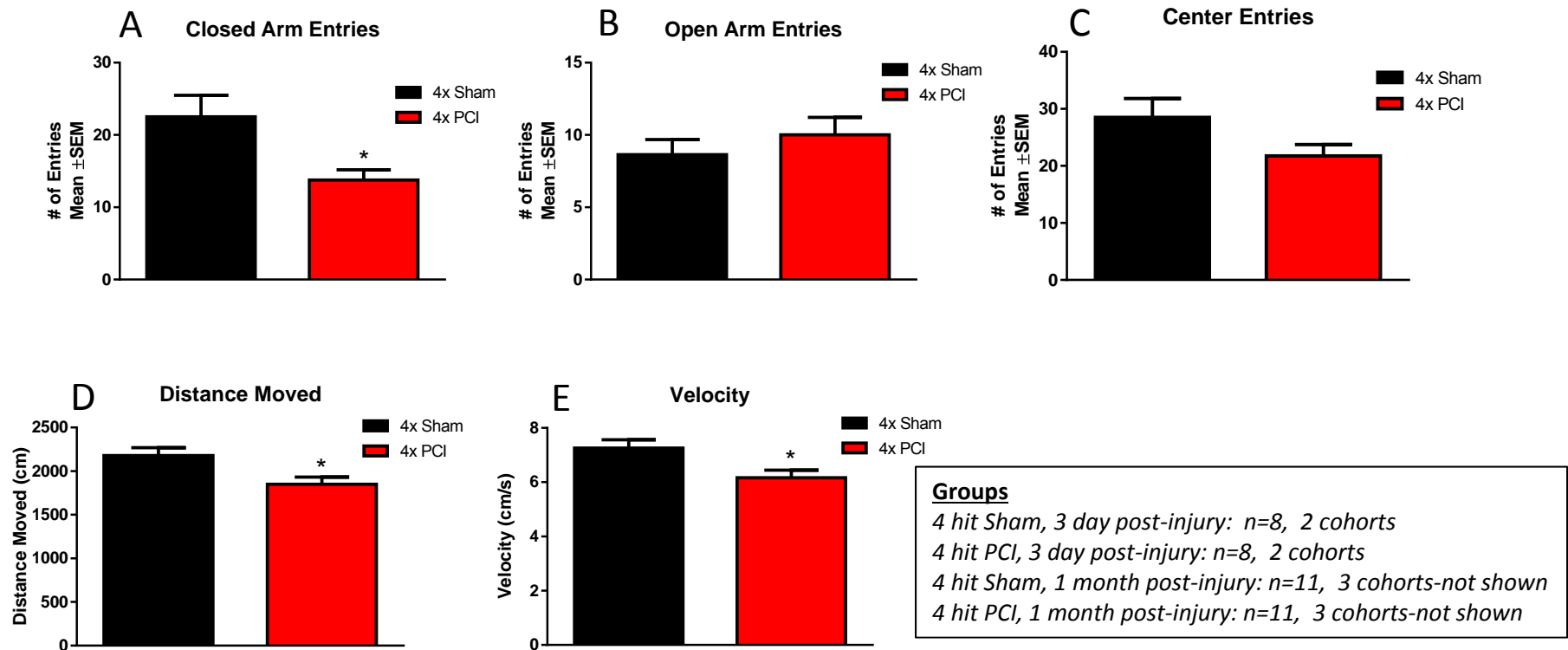


Figure 29. Four hit PCI affects investigation and/or locomotion in the elevated plus maze 3 days post-injury. PCI decreased closed-arm entries compared to sham while having no effect on open arm entries or entries into the center of the maze (A, B, and C). Distance moved and velocity were decreased in the PCI group (D & E). This suggests that PCI may effect locomotion and/or investigation of the maze. Future studies using a multiple testing paradigm will discriminate these possibilities. Note: No significant differences were seen between groups tested at 1 month post-injury (data not shown). * $p < .05$, Student's t-test.

Forced Swim Test: 4 Hit PCI, 1 Week Post-Injury

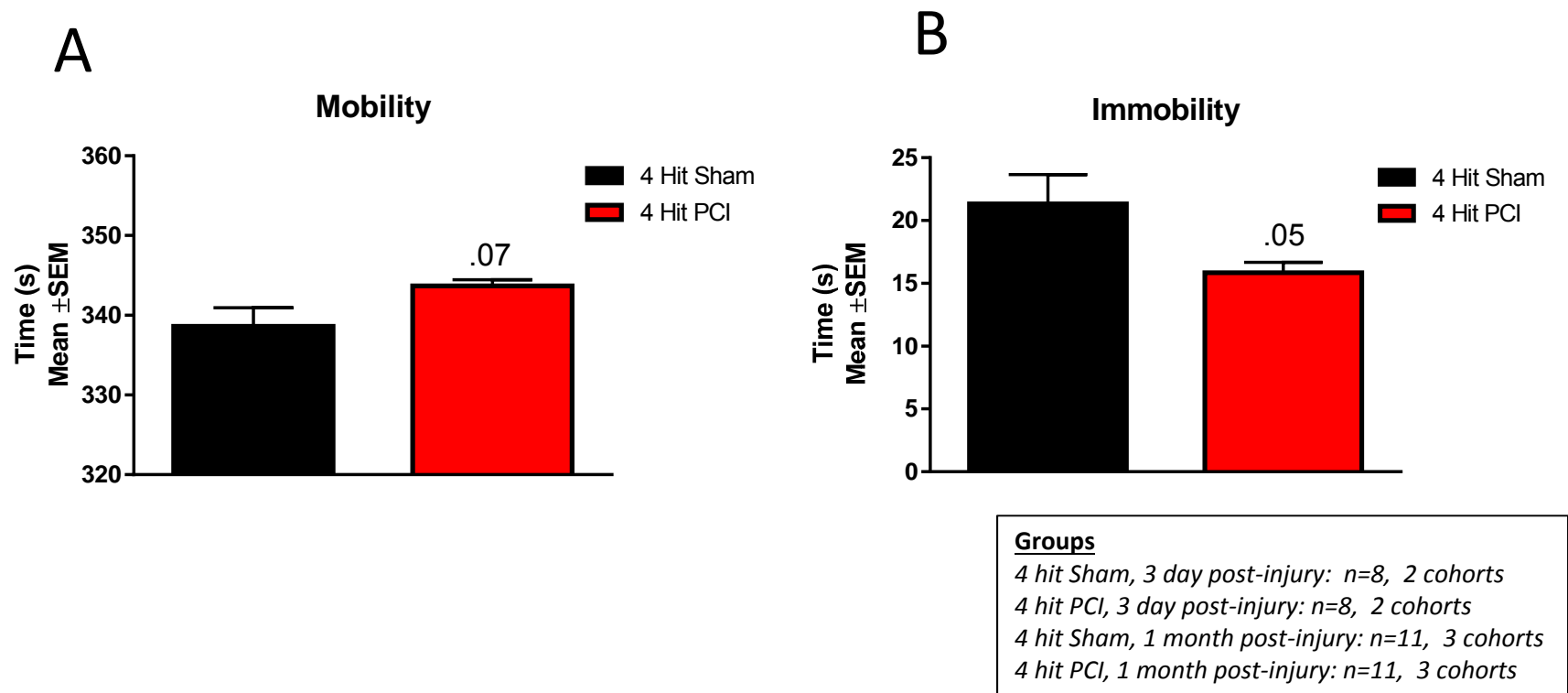


Figure 30. Four hit PCI did not lead to depressive-like behavior in the force swim test. Groups tested one week post-injury showed trends towards increased mobility and decreased immobility in the forced swim test, though these effects were not significant (A&B). The trends show the opposite of the expected effect with a decrease in depressive-like behavior following PCI. No significant effects were observed 1 month post-injury or during the habituation sessions the day prior to testing (data not shown).

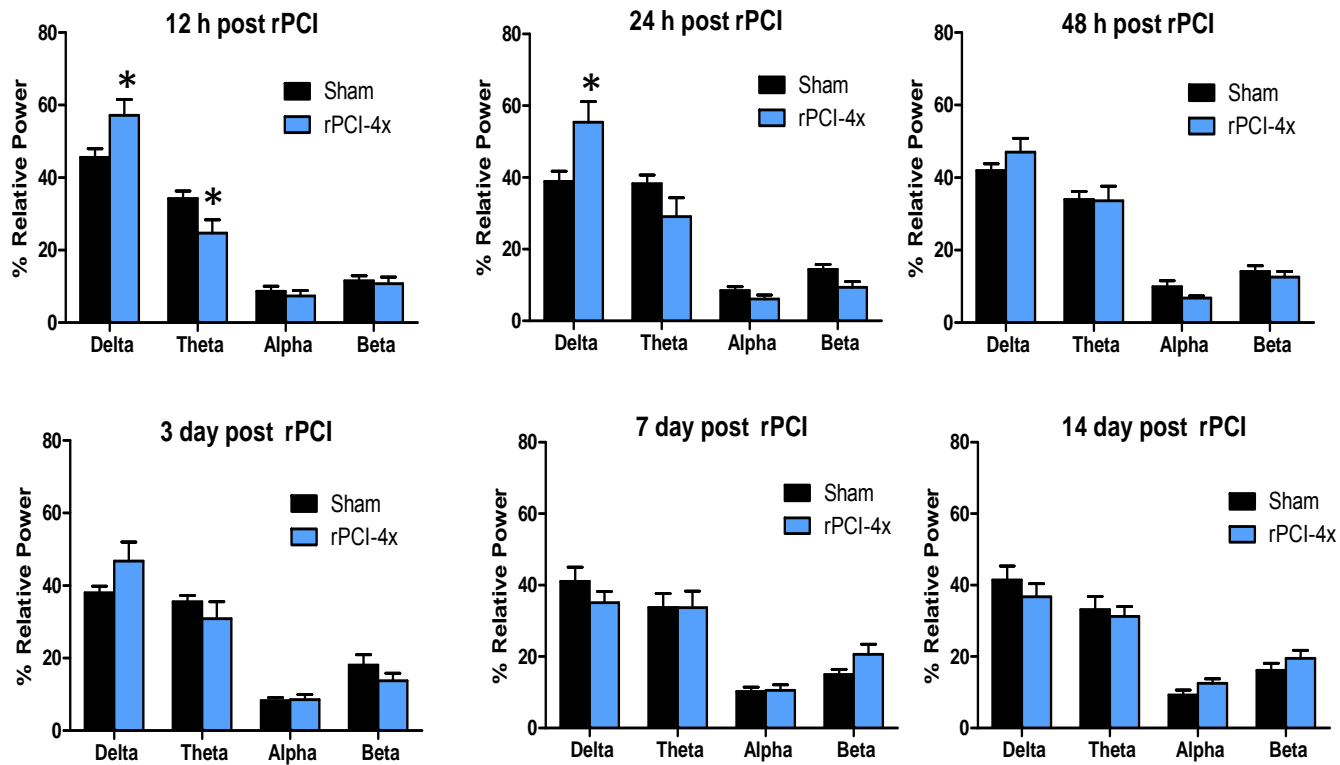


Figure 31. EEG functional changes after repeated PCI: comparison between Sham and PCI-4x groups at each time point .

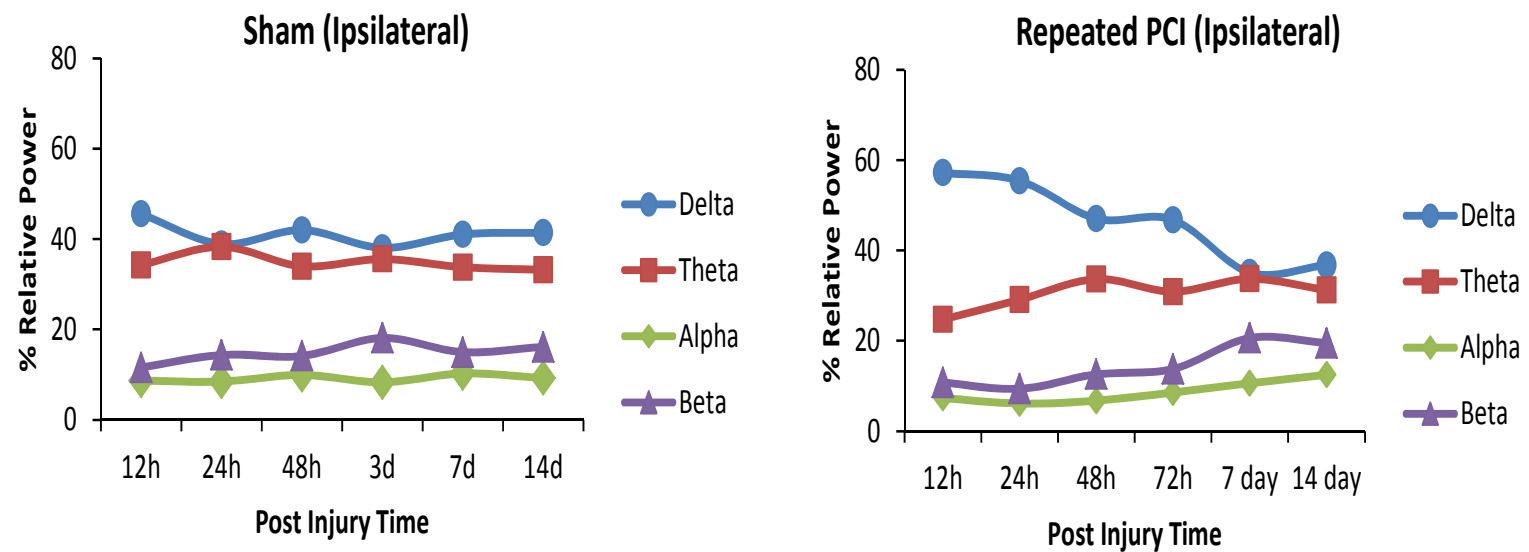


Figure 32. Temporal Profile of EEG functional changes after repeated PCI